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PREVENTION OF HIV TRANSMISSION

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology

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ABSTRACT

Morgan Chateau: HIV Transmission Prevention
(Under the direction of Dr. J. Victor Garcia)

The human immunodeficiency virus (HIV) infects and replicates within individuals for the duration of their life. Initial infection results in little to no symptoms for years or even decades. These individuals are infectious and capable of further spreading HIV while completely unaware of their own status. This ability to transmit without detection is what lead to the unknown and thereby unopposed global spread of HIV type one (HIV-1). Once a test was developed to detect HIV, the virus was found in nearly all major countries around the world. Development of a cure has proven to be exceedingly difficult. So far, the only successful tactic to reduce the number of infected HIV individuals has been to prevent HIV transmission. Traditionally, the most effective way to prevent viral transmission is with a vaccine, but an effective HIV vaccine for wide spread use has not been developed. Therefore, alternative HIV transmission prevention strategies have been used. These strategies largely depend on behavioral modifications and include: 1) utilization of universal precautions in medical settings, 2) increased emphasis on HIV testing and self awareness of infection status, 3) encouraged use of protective measures such as condoms and male circumcision, 4) prophylactic use of antiretroviral drugs to limit mother to child transmission MTCT, and 5) the newly available oral pre-exposure prophylactic use of Truvada in HIV negative individuals. Together, these strategies have contributed to nearly eliminating HIV transmission in medical settings and greatly reduced

MTCT. Unfortunately, HIV continues to spread globally largely due to sexual transmission. While condom usage is highly efficient to prevent transmission, their use is limited by acceptability and consent. In this dissertation, I evaluated the potential of topically applied interventions to be a novel, effective form of protection against HIV transmission as well as established a novel line of investigation evaluating microbial contributions to HIV transmission.

Using humanized mice as a model of HIV transmission, I evaluated two antiretroviral drug based topical pre-exposure prophylaxis (PrEP) for efficacy; tenofovir and maraviroc. In both instances, these drugs were found to be protective when used prior to HIV exposure. Concerns regarding the dual use of tenofovir for treatment as well as PrEP prompted me to evaluate transmission of a tenofovir resistant strain of HIV. This study demonstrated a surprisingly large defect in transmission for tenofovir resistant HIV, which suggests that use of Tenofovir for PrEP may not result in a significant increase of circulating tenofovir resistant strains of HIV.

I also utilized the humanized mouse model to start a completely novel line of investigation evaluating the effect of microbial populations on HIV transmission. While these studies are ongoing, preliminary results have shown a clear effect of microbiome composition on rectal HIV transmission. These results are significant in two ways. First, this is the first evidence that humanized mice are viable tools for microbiome based studies. Second, commensal microbiota does affect HIV transmission efficiency.

Taken together, the following dissertation supports further efforts to curb the HIV epidemic by development of topical interventions (microbicides) and lends credence toward interventions based on commensal microbiome manipulations.

To my parents, my academic fellows, my mentor, and all the mice.

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CHAPTER I. INTRODUCTION

HIV PREVENTION: STRATEGIES CURRENTLY IN USE

The greatest progress to stemming the HIV/AIDS epidemic has been to prevent HIV transmission. There are three main routes HIV transmission can occur: 1) wound/intravenous, 2) mother-to-child, and 3) sexual contact. Of these routes, sexual transmission of HIV is the greatest driving force of the epidemic.

Preventing Sexual HIV Transmission

There were 2.3 million new HIV infections in 2012 [1]. The majority of these transmissions were the result of sexual exposure to HIV. Preventative methods such as the use of condoms, male circumcision, and attempts to modify behavior through awareness and education have been used to help limit HIV transmission through sexual contact [1-3] .

With the exception of abstinence, the most efficient method to prevent sexual HIV transmission is by proper condom use [4]. Male condoms that are used correctly are 95% effective at preventing HIV transmission [4]. Since male condoms are often not used correctly, the average efficacy drops to 70% prevention of HIV transmission [5]. In addition to preventing HIV transmission, condoms can reduce the transmission of other sexually transmitted diseases such as gonorrhea [6, 7]. Factors that limit effective condom use include 1) improper technique, 2) availability of quality condoms that are affordable, and 3) having a partner willing to use a condom. Improper condom use includes failure to keep condom on for the entire duration of the

sexual encounter, incorrect sizing or placement resulting in slippage, and failure to use a condom for every sexual encounter [6]. Availability of quality, non-expired condoms at an affordable price is a major concern in underdeveloped nations [8]. Ultimately, condom use in both under developed and fully developed nations requires male partner acceptance . A study done at an urban clinic in the U.S. reported that 33% of male patients receiving treatment for a STD did not feel motivated to use a condom consistently [7]. Studies investigating condom usage among sex workers have shown an increase in consistent condom usage with clients but a disappointing lack of condom usage with non-paying, regular partners such as boyfriends and husbands [9].

An additional biomedical intervention found to help reduce the HIV transmission risk is male circumcision. The ANRS 1265 trial done in South Africa reported a 60% protection in men who underwent circumcision [10]. A second trial of 2784 men in Kenya found the same level of protection [11]. A third clinical trial in Uganda also found male circumcision to be 55% protective for men with circumcisions [12]. The results of these three trials in addition to other studies prompted the WHO and UNAIDS to jointly recommend male circumcision as an effective prevention for HIV transmission to men although consistent, proper condom use strongly encouraged [13]. Unfortunately, male to female transmission is not reduced by male circumcision.

While each of these methods presented in this chapter have shown progress, they are male/penetrative partner controlled and largely limited by acceptability. Therefore, great efforts are in progress to develop alternative, potentially more acceptable methods for HIV prevention that can be used by the female or receptive partner.

HIV TRANSMISSION PREVENTION: ANTIRETROVIRAL BASED STRATAEGIES UNDER INVESTIGATION

For several years, abstinence, condom use, and male circumcision were the main methods available to prevent sexual transmission of HIV. Over the past decade, several antiretroviral dug based strategies have been under investigation for effective HIV prevention. In general, these strategies can be divided by route of administration; oral, topical, or injectable.

Treatment as Prevention

Current NIH guidelines support the initiation of ART at any stage of HIV infection but places especially strong recommendation to start ART when CD4 cell counts drop below 500 cells/mm^3. An HIV infected individual could delay starting ART for years so long as their CD4+ T cell count remains high enough to result in an effective immune response, thereby delaying the inevitable pill burden and possible complications of being on ART. This makes sense from the individual treatment perspective, but in terms of public health, initiating ART early could help reduce the number of HIV transmissions in the population.

In 2011, the results of the HIV Prevention Trials Network (HPTN) 052 clinical trial showed a 96% reduction in HIV transmission between sero-discordant couples when the HIV infected partner started ART early [14]. This study provided the key scientific evidence needed to encourage "treatment as prevention" strategies [15]. A key component for treatment as prevention is the HIV positive person must be diagnosed. Unfortunately, of the HIV-infected people in the USA, only 4 of 5 are aware they are infected [16]. Therefore, for treatment as prevention to be effective against the HIV/AIDS epidemic a greater emphasis on HIV testing with linkage to care will be necessary.

Oral PrEP

In 2012, the first drug-based HIV prevention option, Truvada, was approved for prophylactic use in the U.S. [17]. Truvada is a single pill containing two drugs; tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC). Truvada has been and still is heavily prescribed to HIV-infected patients as part of their first-line therapy. Its approval for prophylactic use was based on results of the iPREX, Partners PrEP, and TDF2 clinical trials where oral use of Truvada in HIV-negative individuals resulted in reduced transmission of HIV [18-20]. Rates of protection increased when adherence was taken into account [18, 21]. In the iPREX clinical trial, Truvada prevented 47% of infections when given to high risk HIV negative men who have sex with men [19]. This protection rose to 90% when investigators excluded participants who did not have detectable drug in their blood samples collected during study visits [19]. Therefore, participants who actually took the drug as prescribed had a very high level of protection from HIV infection. Partners PrEP reported a 75% protection when Truvada was prescribed to the HIV-negative individual in a serodicordant couple [22]. Adherence was very high in this study, 97%, which investigators attribute to co-counseling of the couples, awareness of partner's HIV infection, and the tendency for individuals in a relationship to encourage dosing [22, 23]. The TDF2 trial enrolled sexually active men and women in Botswana and reported a 62% protection for those who were instructed to take daily Truvada [24]. A fourth major clinical trial evaluating Truvada for PrEP, FEM-PREP, did not show protection but was terminated early due to poor adherence [25].

The failure of FEM-PREP highlights a very important issue. The efficacy of oral PrEP strategies is greatly undermined by poor adherence [23]. Adherence, as determined by detectable

drug levels in blood samples, was highest in the Partners PrEP study (≈90%) and lowest in FEM-PREP (≈30%) [22, 24]. One theory to explain this vast difference in adherence is that participants had a very different perception of the possibility of HIV infection. Participants in the Partners PrEP study were chosen because they were sexually active with a partner who was known to be HIV positive. Participants believed themselves to be at high risk of HIV acquisition. This is a stark contrast to the FEM PREP study where 70% of the participants felt they were "at little to no risk" of acquiring HIV [26].

The efficacy found in iPREX, Partners PrEP, and TDF2 was sufficient for the FDA to approve Truvada for use as PrEP. Unfortunately, prescription of Truvada for use as PrEP has been disappointingly low. Reports have suggested several factors limiting the number of prescriptions for Truvada as PrEP. Despite the release of guidelines by the CDC, clinicians have demonstrated considerable variability in their opinions and decision to prescribe [27]. Despite the availability of official sources of information, at risk individuals may be unaware of the availability of Truvada for PrEP, miss-informed regarding its prescription, or simply unaware that they are "at-risk". In addition, stigma also plays a strong role. Critics of Truvada as PrEP have argued that prescribing HIV PrEP will result in greater promiscuity. Supporters of Truvada as PrEP argue that prescribing HIV PrEP is an act of being responsible for one's own health. It may take several years for the medical and general population to support Truvada as PrEP.

TOPICAL PREP/ MICROBICIDES

Topical PrEP or microbicides are a gel, liquid, foam, or dissolving sheet designed to be used vaginally and/or rectally to prevent HIV transmission. There are multiple reasons why microbicides are attractive as tools for HIV prevention: (i) microbicides are user controlled [28];

(iv) microbicides are predicted to be cost-effective [29, 30] local administration of an antiretroviral gel at the site of exposure will result in higher drug levels at the intended anatomical location than can be achieved using oral PrEP [28, 31-33] while reducing the likelihood of experiencing systemic dosing-associated toxicities [33, 34]; (ii) the reduced toxicity associated with topical microbicides is expected to increase adherence [35]; (iii); (v) topical microbicides can be developed with combinations of viral inhibitors [36]; (vi) an ideal microbicide would be safe and effective in both rectal and vaginal compartments [37-39]; and (vii) antiviral microbicides may also protect against viruses other than HIV (e.g. herpes simplex) [40, 41].

Topical Microbicides that Failed: what can we learn?

Clinical trials evaluating the use of topical microbicides to prevent HIV transmission have shown mixed results. Initial clinical trials were focused on utilizing spermicidal agents to prevent vaginal transmission of HIV while also preventing pregnancy. Nonoxynal-9 (N-9) is a detergent-type vaginal spermicide available without a prescription. Clinical trials of N-9 found an average 75% effective at preventing pregnancy [42-44]. N-9's mechanism of action for preventing impregnation is to destroy a lipid membrane, the acrosome, of sperm cells thereby rendering them immobile. In theory, investigators believed this same ability to break down lipid membranes would affect the lipid membrane surrounding the HIV virion thereby rendering the particle unable to bind/merge with a target cell [45]. In 1985 and 1988, N-9 was reported to be effective at reducing HIV infection in *in vitro* [46, 47]. Based on these results, several investigators recommended the use of N-9 to prevent HIV transmission to women [48-50]. In a clinical trial of commercial sex workers in Nairobi, the women who used N-9 containing sponges

had a 10% higher rate of HIV acquisition compared to the placebo group [51]. This increase in HIV acquisition may have been due to the epithelial damage experienced with N-9 use resulting in increased frequency of genital ulcers and vulvitis reported with N-9 use [51, 52]. This exfoliating effect of N-9 was also observed when N-9 containing products are applied rectally and also results in increased viral transmission [53-56]. Therefore, N-9 has been discarded as completely unsuitable for topical HIV prevention [55]. This study has made it clear that future microbicides must not induce breaks in epithelial integrity or induce an inflammatory response.

Alternative "first-generation" microbicides that have undergone Phase I/II safety and tolerability studies in HIV-uninfected and/or HIV-infected volunteers include polymeric viral fusion inhibitors (dextran sulfate/Emmelle, carrageenans [PC-213, PC-503, PC-515/Carraguard], cellulose sulfate/Ushercell, polystyrene sulfonate, naphthalene sulfonate [PIC 024-4/PRO 2000/5], acidifying gel [Carbomer 974P/BufferGel], Lactobacillus (L. crispatus) suppository/CTV-05, detergent-type dual-function barriers [ACIDFORM, GEDA Plus, SURETE, Glyminox/C31G/Savvy, Invisible Condom], and herbal extracts [Praneem] [57]. While all of these microbicides were proven effective *in vitro* or in cell culture, none of them passed human efficacy trials.

Much like N-9, cellulose sulfate based microbicides, like Ushercell, were developed to act as both a birth control and protection against STD acquisition [58]. Unlike N-9, Ushercell did not induce epithelial sloughing or ulcers. Cellulose sulfate is a polyanionic compound from the carrageenan family that binds charged surfaces in a non-specific manor. These types of molecules are used by many microbes for initial attachment to cell membranes. In theory, a gel with cellulose sulfate will result in sperm and infectious virions binding to the compounds in the gel rather than to target cells [59, 60]. In the rabbit model, Ushercell was found to be an effective

contraceptive capable of preventing 95-100% of pregnancies [58]. This same paper reported Ushercell to be non-toxic to host cells or to *Lactobacilli* growth [58]. In vitro, Ushercell had several anti-microbial effects on herpes viruses, IC_{50} = 59ng/mL for HSV-1 and IC_{50}= 24 ng/mL for HSV-2, on HIV, IC_{50} between 3 and 78 ug/mL for various HIV strains, on Neisseria gonorrhoeae, IC_{50}= 2 ug/mL, and on Chlamydia trachomatic, IC_{50} = 78 ug/mL [58]. Multiple phase I and II safety trials were done for vaginal application of Ushercell and reported no safety concerns [61, 62]. One study evaluated Ushercell use in HIV infected women. While the investigators concluded there to be no safety issue, it should be noted that 70% of study participants reported minor to mild adverse events in both the experimental and the control arms of the study [62]. Another study evaluated the safety of Ushercell when applied to the penis of HIV infected men [63]. In this study, the investigators reported the gel to be "well tolerated" despite 42% of Ushercell users reporting adverse events such as itching, burning, testicular pain, dysuria, and warm/cold feelings [63]. In 2007, two phase III trials evaluating cellulose sulfate based microbicides were terminated early due to an increased risk of HIV acquisition in participants using the cellulose sulphate gel, Ushercell. In one of these studies, the Ushercell gel was being tested on 1333 women in Benin, India, South Africa, and Uganda as collaboration with Conrad, the Eastern Virginia Medical School, and the US Agency for International Development with funding from the Bill and Melinda Gates Foundation [64]. The second study was being conducted by Family Health International in Nigeria on 1800 women [64]. At the time, the FHI study did not have an increase HIV risk with Ushercell, but the study was stopped given the preliminary results in the Conrad trial [64]. What data was collected from the Conrad trial indicated that Ushercell was not preventing HIV transmission and that overall rate of adherence to gel was low; 50% of sexual encounters without a condom [65].

Dextran/dextrin sulfate is another polyanionic compound that was found effective in cell culture HIV protection assays [66]. Unfortunately, it is not absorbed well when given in oral dose [67]. To investigate the anti-HIV potential of dextran sulfate, one study administered the drug via continual intravenous dosing to HIV positive patients [68]. Despite achieving plasma drug levels 200 fold greater than the in vitro IC50, the patients exhibited an increase in detectable viral p24 [68]. In addition, continual dosing resulted in high toxicity [68]. Topical gel formulation of dextrin sulfate (Emmelle) was found to be safe when applied to the vagina or the penis of healthy volunteers [69-71]. Before starting a Phase III efficacy trial under the UK's Microbicides Development Programme (MDP), ML laboratories , the makers of Emmelle, announced that they would abandon development of Emmelle in favor of a newer, more potent microbicide, PRO 2000 [72].

PR0 2000 was a naphthalene sulfonate polymer containing gel developed by Interneuron. This polyanionic compound had been shown to bind directly to the HIV gp120 protein and interfere with viral attachment/fusion [73]. In tissue culture studies PRO 2000 was found to be highly potent and did not generate a cytotoxic effect [74]. As mentioned above, PRO 2000 was evaluated for efficacy in a MDP clinical study [75]. MDP-301 was a three arm study performed in South Africa, Tanzania, Uganda and Zambia. Women were randomly assigned to receive either a 0.5% PRO2000, 2.0% PRO2000, or a placebo gel and instructed to use the gel (available in single use applicators) within one hour before sexual intercourse [75]. Participants were followed for 52 weeks and evaluated for HIV status. While the gel was well tolerated, it was completely ineffective at preventing HIV transmission and the trial was terminated early [75, 76]. Despite this, interesting data was collected regarding adherence. Adherence to orally administered drugs can be estimated by the presence of drug in the blood. Adherence to topically

administered interventions may not result in detectable levels of drug in the blood stream. In this clinical trial, in addition to self-reporting gel use, women were asked to bring one used applicator to each study visit. These applicators were then stained using a 1% trypan blue solution and evaluated for whether or not they were vaginally inserted [77]. This test had previously been found to be fairly accurate and offered a novel aspect to the MDP-301 study [77]. Participants in the MDP-301 study were only considered to have used the gel consistently if they filled three criterion i) self-reported consistent gel use, ii) presented a used vaginal applicator at each study visit, and iii) attendance of at least half of all study visits. Adherence, and how it is determined, will play a large role in clinical study design and interpretation of results. MDP-301 was one of the first topical microbicide trials to measure adherence using a criterion beyond self-reporting and applicator count.

Carraguard (PC-515) was developed by the Population Council in New York and, much like PRO2000, was designed to inhibit viral binding/fusion to target cells. In 2008, the results of a clinical trial involving over 6,000 participants found Carraguard to be safe but ineffective at preventing HIV transmission [78]. While this study did not find efficacy, it provided useful information regarding acceptance and adherence. In this study, self-reporting of gel use was very high (96.2% Carraguard arm, 95.9% placebo arm) but analysis of used applicators estimated a far lower adherence (41.1% Carraguard arm, 43.1% placebo arm) [78]. This is a lower adherence that what was reported for the previous MDP-301 study. It has been suggested that adherence was higher in MDP-301 due to the recruitment of sero-disordant couples where the study participant is fully aware of their partner's HIV infection.

BufferGel is a product designed to maintain an acidic environment in the vagina. A pH value around 4 is considered healthy for the vaginal cavity and may kill or inactivate potential

pathogens. During sexual acts, the alkalinity of semen may temporarily raise the pH thereby removing some of the antimicrobial protection. BufferGel was tested for safety and efficacy in multiple animal models. When used in rabbits, BufferGel was an effective contraceptive and reduced transmission of rabbit papillomavirus [79]. When used in mice, BufferGel prevented vaginal transmission of Chlamydia trachomatis but did not prevent transmission of Neisseria gonorrhoeae [79]. When tested vaginally and rectally in macaques, Buffer Gel was found to be overall safe and did not affect microflora but did result in epithelial desquamination when used rectally [80]. This same study reported that BufferGel did not prevent cervical or rectal chlamydial infection.[80] A safety study found daily administration to the penis of HIV negative or HIV-positive men resulted in no serious adverse events which supported progressing to efficacy trials in women [81] A safety study in India, Thailand, Malawi, and Zimbabwe asked participants to apply BufferGel vaginally twice per day for two weeks [82]. In this trial, BufferGel did not disrupt the vaginal epithelial layer and treatment resulted in reduced prevalence of bacterial vaginosis from 30% at enrolment to 6% [82]. Unfortunately, BufferGel was found to have no effect on HIV transmission to women [83].

While none of the previously mentioned microbicides were effective at preventing HIV transmission, their clinical trials highlighted two main issues that need to be considered before starting another phase III trial. First, greater attention and more diligence is needed to establish microbicide safety before moving on to efficacy trials. This can be addressed with more rigorous animal testing and a stricter definition for “well tolerated”. A second issue for microbicide research is adherence. Several of the clinical trials summarized here utilized various definitions of “adherent” which makes cross comparison between trials difficult. In some cases, the clinical trial itself reported low adherence. While some trials such as the MDP-301 made a concerted

effort to discover factors affecting adherence (housing conditions), this information should be part of every microbicide trial.

Topical Microbicides that Succeeded: What can we learn?

The greatest successes have been with antiretroviral based topical microbicides. Of the antiretroviral drugs being evaluated, tenofovir based microbicides have shown the greatest promise. The CAPRISA004 study evaluated the use of a 1% tenofovir gel to prevent transmission of HIV in women who were instructed to apply the gel vaginally within twelve hours before sex and to apply a second dose within twelve hours after exposure. Overall protection was 39% and this increased to 53% in women with high adherence [40]. This was the first clinical trial to demonstrate protection using a microbicide.

Rectal Microbicide Development

Based on the CAPRISA004 success, the same drug formulation was used for clinical evaluation to prevent rectal HIV transmission, MTN-006. Unfortunately, the formulation induced unpredicted side effects, low adherence, and several reported adverse events such as diarrhea, nausea, bloating, and pain [84]. It was thought that the side effects were due to the high osmolarity of the gel formulation [84]. Results of this trial highlighted the need for development of a rectal specific microbicide or ideally a microbicide safe and effective for both vaginal and rectal use. The following clinical trial, MTN-007, was a phase I safety and acceptability trial designed to test a new formulation of the 1% tenofovir gel [85]. This new 1% tenofovir gel had a reduced osmolarity and did not cause the gastrointestinal side effect reported in MTN-006 [85].

This new, rectal safe gel is currently under investigation for HIV prevention efficacy in the MTN-017 clinical trial.

Further trials are needed to evaluate dosing strategies and optimal formulations as well as potential alternative antiretroviral drug candidates, but the future for microbicide development is encouraging. Evaluation of potential antiretroviral drugs with various dosing strategies and optimization can be evaluated, pre-clinically, using animal models.

DRUG DISTRIBUTION FOR ORAL VS TOPICAL ANTIRETROVIRAL ADMINISTRATION

PrEP is being evaluated in three main routes of administration 1) oral, 2) topical, and/or 3) injection. While several antiviral compounds have been investigated, the ones furthest along in development are antiretroviral drugs that have been approved for use as ART. Of these drugs, tenofovir (TDF or TFV) and emtricitabine have been characterized the most and will be the focus of the following sections.

Oral administration of PrEP

Currently, ART drugs are prescribed as one or more pills to be taken orally. For the purposes of PrEP, an ideal antiretroviral drug candidate should have high penetration in tissues where HIV is likely to transmit (cervix, vagina, penis, rectum). Antiretroviral drugs from the protease inhibitor class only reach concentrations in the female genital tract that are less than half of what is in the plasma [86]. Drugs from the nucleoside reverse transcriptase inhibitors can reach two to six fold higher concentrations in mucosal tissues than in plasma [86]. In one study,

healthy volunteers took 300mg of Maraviroc twice a day for seven days [87]. The area under the drug concentration curves reported a 1.9 fold increase in vaginal tissue and a 2.7 fold increase in cervicovaginal fluid compared to blood plasma levels [87]. The integrase inhibitor raltegravir has similar levels, multiple dosing resulting in CVF drug concentrations two fold greater than plasma [88].

As mentioned in a previous section, Truvada has been approved for use as oral PrEP. Maraviroc is also under evaluation for both oral and topical PrEP. Therefore, this section will describe the reported drug distributions of tenofovir, emtricitabine, and maraviroc. For oral administration, tenofovir (TFV) is formulated as a tenofovir disproxyl fumerate (TDF). This prodrug form of tenofovir is readily absorbed and disassociates into TFV which is then converted to the active drug form within the cell. Under steady-state conditions (regular oral dosing), the area under the curve values for TFV are 4 fold higher in vaginal tissue and 33 fold higher in rectal tissues than in blood plasma [89]. Under steady-state conditions, emtricitabine reaches values 4 fold higher in vaginal tissues and 4.4 fold higher in rectal tissues than in blood plasma [89]. Maraviroc reaches 4 fold higher in vaginal tissues and 27 fold higher in rectal tissues [89].

Oral administration of antiretroviral drugs does not always result in uniform drug distribution to tissues. All three drugs achieved drug concentrations in tissues that are greater than in blood plasma Tenofovir and maraviroc attained higher drug concentrations in rectal tissue than what is achieved vaginally. Emtricitabine achieves roughly the same concentration in both rectal or vaginal mucosal sites. These differences in drug distribution have been considered as possible contributions to the different levels of protection measured in iPREX, a MSM protection study, and in FEM-PrEP, a heterosexual women protection study . On the other hand, Partners PrEP and TDF2 showed protection regardless of participant gender.

Topical Administration of PrEP

Topically administered PrEP comes in several forms 1) gels, 2) liquids, 3) disovable sheets, 4) dissolvable pills/suppositories, 5) vaginal rings, 6) drug containing sponges, 7)aerosols/nebulizers, 8) powders, and other creative options. For this section, I will focus on the forms which have been most frequently studied: gels, liquids, dissolvable solids, and vaginal rings.

Gels and liquids are popular options due to their ease of use and similarity to sexual lubricants and spermicides. They have the potential to release a large amount of drug in a short period of time (ideal for peri-coital dosing) or can be designed to slowly release a constant amount of drug over longer periods of time (ideal for daily dosing). MTN-001 was a three arm clinical trial evaluating the pharmacokinetics of a 1% tenofovir gel applied vaginally (40mg) and/or orally administered tenofovir (300mg) [90]. Results from this study reported that serum concentrations after vaginal dosing were 56-fold lower than oral dosing but that vaginal tissue tenofovir diphosphate was 130-fold higher than in oral dosing [90]. Another curious result was that rectal fluid tenofovir concentrations in vaginally dosed participants were higher than concentrations measured in the orally dosed arm. This would suggest that vaginal application of tenofovir results in some drug migration to the rectum. A similar effect was measured in macaques where rectal fluid TFV concentrations were a log lower than vaginal fluids after vaginal dosing [91]. RMP-02/MTN-006 evaluated the pharmacokinetics of a 1% tenofovir gel applied rectally as compared to orally administered tenofovir [84]. Unfortunately, the gel formulation evaluated was not optimized for rectal use and resulted in several adverse events

[33]. The pK data gathered from this study demonstrated that topically applied gel resulted in maximum tissue drug levels in less than 30 minutes and these levels were 6-10 times greater than the maximum levels oral drug administration achieved [84].

Dissolvable solids such as sheets and pills have been used previously for distribution of contraception (vaginal) or laxatives or opiates (rectal). These devices are designed to release a large amount of drug in a short period of time. A study done in pigtail macaques showed that a vaginally applied rapidly disintegrating tablet containing tenofovir (10mg/pill) or tenofovir and emtricitabine (10mg each) was able to dissolve in less that 30mins with no effect on local inflammatory cytokines, pH or microbiota [92]. Investigators found peak vaginal concentration around the 10^4-10^5 ng/g tissue (147-571 uM) range at 4hr post dose with sustained levels 24hrs post dose [92]. Similar levels were reported for FTC. These concentrations are predicted to be protective based on previous studies [93, 94].

Vaginal rings are commonly used for contraception and function by the device slowly releasing a constant amount of drug for several days (usually a month). Unlike gels and dissolvable solids, drug releasing rings may not be practical for rectal use. While vaginal rings designed to elute antiretroviral drugs have not been tried in humans, they have shown very promising results in macaque models [95]. Smith *et.al.* tested TDF-eluting vaginal rings in macaques [95]. Each ring eluted TDF continuously for 28 days and was then replaced with a new ring for a total of 4 months of drug/ring exposure. After 16 weekly vaginal exposures to SHIV162p3, all of the animals with TDF rings remained non-infected while 11 out of 12 control animals became infected [95]. Similar to the pharmacokinetic results seen in gels and dissolving solids, the rings generated high cervical fluid levels of tenofovir (mean 1.8x10^5 ng/mL) [95]. Preliminary acceptability trials using placebo rings in Africa reported that women found vaginal

rings to be highly acceptable once initial concerns such as the ring “getting lost inside the body” (20% of participants) were addressed [96].

In general, topical administration of antiretroviral drugs results in highest drug levels where the PrEP is administered. Depending on adsorption, some drugs applied topically can be detected in plasma and other tissues but these levels may not offer protection. There are many options for topical administration of PrEP which may improve adherence.

Long acting injections for PrEP

Long-acting injections of antiretroviral drugs have great potential for both ART and PrEP. In theory, a patient would be able to receive an injection of drugs once a month or once a quarter instead of taking daily pills. This technology has been safely used for administration of common methods of birth control, Depo-Provera and DMPA, in many sub Saharan countries where HIV is most prevalent [97]. In terms of PrEP, long acting injections could greatly improve adherence by providing a consistent, systemic level of drug release over an extended period of time [98-100]. At this time, these anti-HIV injections have only been tested using animal models but the results are very encouraging [99-101]. In a recent study published in *Science*, a novel long-acting injectable integrase inhibitor was administered to macaques at two time points 4 weeks apart [101]. These animals were challenged weekly for 8 weeks and remained non-infected [101]. The same study also did an experiment were animals were give a single drug infection followed by weekly exposures to virus until the animals became infected [101]. The animals remained protected from infection until plasma drug levels dropped several weeks after initial drug administration [101].

Topical vs Oral PrEP

Topically administered PrEP can achieve higher tissue drug levels than has been achieved with oral PrE . Topically administered PrEP is most effective at the site of application. Formulations for topically administered PrEP need to be designed to function in both vaginal and rectal compartments.

Drug formulations for oral PrEP is much simpler since the oral preparations used for ART already have passed safety evaluations and gained FDA approval. Orally administered PrEP can provide systemic distribution of drugs which may result in HIV protection from multiple routes of exposure including from injection drug use.

Overall, it is unknown what concentration of drug is needed for protection at the different sites of mucosal exposure. More detailed studies will be needed to determine what the minimum amount of drug presence is needed to provide significant protection. The only assumption that can be inferred is that “more is better”. In other words, we know that a complete absence of drug presence will not provide protection. On the other hand, several clinical trials correlate drug presence with protection.

Ideally, topical, oral and injectable forms of PrEP should be developed and approved for use. Having multiple options available for PrEP may encourage adherence to use at least one if not more methods to prevent HIV transmission.

DRUG SUSCEPTIBILITY AND RESISTANCE

“Drug susceptibility” refers to the amount of drug needed to suppress viral replication *in vitro*. Drug susceptibility testing is usually done by culturing a fixed viral inoculum with a serial dilution of a viral inhibitor and measuring some output signal for viral replication (p24, copy

number, fluorescent indicator, etc). The concentration of inhibitor needed to prevent 50% of in vitro replication is the $IC_{50.}$ The choice of culture cells, viral inoculum, indicator for viral replication, and other parameters can result in different IC_{50} values unique to that particular assay and to the specific viral inhibitor used. Therefore, comparisons of IC_{50} values can only be done if the assay used to generate them are similar.

While drug susceptibility testing cannot be used to predict the amount of drug needed for *in vivo* treatment, it is a powerful tool for evaluating relative drug susceptibilities for genetically different viral strains. These differences in susceptibilities (often expressed as fold change or "shift" in IC_{50}) have been used to determine which genetic mutations contribute to drug resistance. For example, the K65R mutation in HIV's reverse transcriptase protein will result in a 2 to 4 fold increase in tenofovir $IC_{50.}$ This means, a virus containing the K65R mutation is expected to require 2 to 4 times more drug to reach the same suppression seen in a virus with the original lysine residue. It should be noted that drug resistance is usually referring to fold changes in IC_{50} as a result of one or a few mutations difference from an original "wild type" sequence. This is different from the natural variation in IC_{50} values observed between strains of HIV who would each be considered "wild type". This concept will be explored more in the following sections.

Drug Resistance Based on Genotype

As mentioned before, HIV has an incredible amount of natural diversity and variation in genetic sequence which makes defining a "wild type" sequence difficult. Fortunately, advances in gene sequencing technology and the creation of large sequence databases such as those provided by the NIH and the Stanford Drug Resistance Database and have allowed researchers to

compile phenotypic information as they relate to specific genotypic sequences. Not only have consensus sequences been developed for all major HIV clades, specific variations from these consensus sequences have been identified as contributing to drug resistant phenotypes. Using the same example from the previous section, the K65R mutation represents a single amino acid variation from consensus sequence that has been shown to decrease susceptibility to tenofovir. Therefore, sequence data obtained from patients can be compared to the consensus (wild type) sequences available for each HIV clade.

Variations between patient sequence and wild type sequence, also known as polymorphisms, are common but not all variations contribute to drug resistance. To simplify matters, the WHO and the IAS publish yearly updates listing which mutations are considered to be “drug resistance” mutations. It should be noted thought that variation in viral polymorphisms can often reduce or increase the effect of a known drug resistance mutation [102].

De novo drug resistance and replicative fitness

HIV can develop resistance to all drugs currently available for treatment [103, 104]. The mechanism for development drug resistance lies in the high error rate of HIV’s reverse transcriptase. Each round of viral replication can result in one or more nucleotide misincorperations. Some misincorperations will result in a defective viral genome and the progeny virus will be non-infectious or have a reduced replicative capacity. These viral lineages will be rapidly out competed by viral lineages that do not have a defect [105, 106]. Other nucleotide mutations may result in changes of amino acid sequence that confer resistance to antiviral drugs. These mutations are rare but can occur at any time resulting in resistance to any antiretroviral drug [107]. In very rare cases, a single round of replication can result in

accumulation of more than one drug resistance mutation. In the absence of antiretroviral drugs, drug resistant viral genomes do not have an advantage and are therefore unable to out compete the general viral population. In the presence of antiviral drugs, the relative fitness of drug resistant virus is greater than that for drug susceptible virus and the resistant virus will be able to out compete and dominate the infection [105, 106, 108-111]. Therefore, the use of antiretroviral drugs does not create drug resistance mutations in HIV. The mutations occur randomly and selective pressure provided by the presence of an antiretroviral drug is what allows a drug resistant HIV to dominate the infection.

To prevent the out growth of drug resistant HIV, current ART therapies consist of a cocktail of multiple drugs with different classes of action. While it is possible to acquire a drug resistance mutation easily, this mutation is usually only effective against one drug or one class of drugs. For example, the K65R mutation is effective against the NRTI drug tenofovir, but the mutation is not effective against any of the protease inhibitors. Therefore, even if a patient has a viral population with a tenofovir resistance mutation, this population will be suppressed by one of the other drugs being prescribed. It is possible, but less-likely, for a single round of viral replication to result in two drug resistance mutations. For this reason, patients are prescribed at least three different antiretroviral drugs. The likely hood of a single round of viral replication resulting in acquisition of three drug resistance mutations is extremely low. The low "likelihood" of developing drug resistance is often referred to as the genetic barrier to resistance. Several studies have been tried to quantify the genetic barrier to resistance [112-114]. Factors that affect the likelihood of developing a drug resistance mutation include what type of mutation is needed to induce the amino acid change and how many nucleotide mutations are needed [107, 115, 116].

Polymorphisms at other nucleotide locations may also affect the selection of drug resistance mutations [102, 117-119].

Transmitted Drug Resistance

For this section, “drug resistant virus” will be defined as HIV with at least one of the genetic mutations listed on the WHO or IAS drug resistance surveillance reports [103, 120]. Drug-resistant viruses can be transmitted from person to person [121-124] . The inherent ability of replicating HIV to revert to a drug sensitive genotype in the absence of drug pressure makes it difficult to study in patients especially if: (1) the time/duration/route of infection are unknown, (2) there is no way to prove ART naïve status, and (3) the HIV sequence in the infecting partner is unknown. Despite these difficulties, genotypic analysis of ART naïve patients has provided evidence that drug resistant HIV-1 is being transmitted and can result in treatment failure [125-131].

As mentioned before, drug resistant HIV can only be selected for by the presence of antiretroviral drugs. Proper prescription and adherence to ART can prevent out growth and spread of drug resistant HIV. Therefore, it is no surprise that the regions with greatest transmitted drug resistance are areas where antiretroviral drugs have only recently been made available and adherence is not consistent. For example, the past decade has seen a dramatic increase in ART availability in resource limited areas in eastern and southern regions in Africa. along with this increase in ART, there has been an increase in transmitted drug resistant HIV [132].

The frequency of transmitted drug resistance varies depending on the specific drug resistance mutation in question, that mutation’s predicted effect on replicative fitness, and what

antiretroviral drugs are used in the region where transmission is occurring. For example, the most commonly transmitted drug resistance mutations tend to be NRTI or NNRTI mutations due to the high use of these drugs for ART [103, 120, 123, 124, 128, 129, 132]. Of these mutations, K65R and M184V are of specific interest since they confer resistance to the two drugs most heavily investigated for PrEP (tenofovir and emitracitabine).

Effect of PrEP on Transmitted Drug Resistance

The use of first line therapy drugs for both protection and treatment has raised concerns that there will be an increase in transmission of drug resistant HIV. An example of this would be a patient who is taking Truvada as a PrEP but who experiences a break through infection and continues to administer Truvada. Truvada is a dual drug formulation that may not be sufficient to fully suppress HIV infection and the end result is rapid de novo development of drug resistant HIV which can then be passed to future individuals. This exact scenario has occurred in at least one of the oral PrEP clinical trials [22]. While this is a real concern for individuals, it may not result in an increase in transmitted drug resistance at the population level.

In 2010, Supervie *et. al.* published a model which predict that PrEP would reduce the prevalence of drug resistant HIV [133]. This reduction is based on two main predictions. First, use of PrEP will reduce the number of HIV infected individuals, which will inturn reduce the opportunity for *de novo* drug resistant HIV to develop. If there are fewer people who develop drug reistance, then there are fewer opportunities for drug resistant HIV to be transmitted. Supervie finds this to be true even if a percentage of individuals on PrEP develop resistance due to undetected breakthrough infection. The second main prediction assumes that transmission of drug resistant HIV will still be inhibited (at least by some small degree) by PrEP. This prediction

is based on the fact that "drug resistance" is merely a shift in drug susceptibility resulting in only a reduction in drug efficacy rather than a complete immunity to drug effect.

Most models assume that the PrEP being used is an orally administered antiretroviral drug. As described in the previous section, drug distribution patterns are very different between oral and topical administration. How these differences in drug distribution can affect the *de novo* or transmission of drug resistance in a PrEP/microbicide situation will require more study. There is as of yet, no evidence that topically administered antiretroviral PrEP can result in generation of drug resistant HIV. Animal studies could be very useful to evaluate the safety of topical PrEP in infected individuals.

MICROBIOTA EFFECT ON HIV TRANSMISSION

The term "microbiota" refers to a collection of microbial species while the "microbiome" specifically refers to the genetics of those microbial species. There is no realistic way to measure the "microbiota" since most commensal microbiota cannot be cultured and microscopic classification of every species in an individual's microbiota would take decades, but there are ways to evaluate the "microbiome" through high throughput, deep sequencing [134]. Deep sequencing platforms such as the Illumina series and 454 Life Sciences systems allow researchers to determine the composition of a sample's entire microbiome down to the single genome level [135]. These technologies have been used to highlight the importance and involvement of the human microbiome in wellness and disease.

Highly competitive environments such as the gastro-intestinal tract and male and female reproductive tract provide sites for complex microevolution and extensive co-dependence

networks for the microbiota present [136]. The composition of the microbiome can fluctuate greatly and in a short period of time [137-139]. The intestinal tract and reproductive tract of mammals are initially colonized by microbiota from their mother during birth, nursing, and interactions with the environment [140-142]. Through life, the microbiota of the intestinal tract is greatly influenced by host diet [143]. The microbiota of the female and male reproductive tracts are greatly influenced by sexual activity, changes in hormones, as well as cleaning practices [144-146]. Biomedical interventions such as antibiotic treatments, use of diuretics, use of laxatives, anti fungal medications, use of drug based birth controls, and circumcision all have an impact on microbiota composition [147-149]. Majority of the changes to the microbiome go un-noticed by the host organisms, but occasionally changes can result in symptoms. Changes in intestinal microbiome have been associated with irritable bowel disorders such as colitis and Crohn's disease [143, 150]. Patients using large amounts of antibiotics may see rapid out growth of *Clostrida difficile,* which is best treated by re-introduction of commensal organisms who then compete with *C. difficile* for resources [148, 151-153]. Changes in vaginal microbiome can result in a condition known as bacterial vaginosis or yeast infection [154-156].

Microbiome may affect HIV transmission and pathogenesis

Acquisition and pathogenesis of sexually transmitted diseases, such as HIV, may be enhanced or limited by microbiota at the site of mucosal exposure [145, 154, 155, 157]. Specifically, concurrent symptomatic infection of an STD with inflammation, rash, ulcers, or other breaches in the mucosa enhance the probability of HIV transmission [158]. On the other hand, far less is known about the effect of commensal microbiota, but there is a building body of evidence supporting the theory that commensal microbiota also plays a role in HIV acquisition.

Given the inherent tendency for commensal microbiota to rapidly fluctuate in composition within the same individual, studies to evaluate microbial contribution to HIV transmission in humans are often difficult and can only estimate the microbial composition at the estimated time of HIV transmission. Despite this large difficulty, commensal microbiota has been indicated as affecting sexual HIV transmission in several studies [145, 159-162] . A study done in Thailand looked at both HIV negative and HIV positive sex workers [162]. This study found a correlation between vaginosis and HIV infection but could not determine which condition came first. A different study of 657 HIV negative, female sex workers in Kenya examined the relationship between vaginal colonization with lactobacilli, bacterial vaginosis, and poteintial acquisition of HIV [161]. Women who did not vaginal lactobacillus species had an increased risk of acquiring HIV and gonorrhea. Women who had abnormal vaginal flora had an increased risk of both HIV and Trichomonas infection. In general, bacterial vaginosis is presumed to increase acquisition of HIV while lacto bacilli are presumed to decrease acquisition of HIV.

The majority of HIV transmission studies focused on the vaginal econiche. Few studies have been done evaluating the effect of microbiota on rectal HIV transmission and the results were inconclusive [163]. In 1994, Law et. al. published the results of a study evaluating 144 MSM subjects [163]. The study found a correlation between HIV infection and detection of spirochaets in rectal biopsies but could not determine if infection was cause or effect [163].

ANIMAL MODELS TO TEST HIV PREVENTION

SIV/Non-human Primate Models

The safety and efficacy of antiretroviral drugs can be evaluated in animal models before their use in human clinical trials. HIV represents a unique difficulty due to its limited tropism. In 1986 the etiological agent for AIDS was isolated and its discovery gave scientists the ability to start empirical transmission studies. One year after the discovery of HIV-1, chimpanzees were utilized as a model to study HIV-1 transmission because they are the only animals, other than humans, naturally capable of sustaining an HIV infection. Chimpanzee studies improved our knowledge of HIV-1 transmission and pathogenesis but had noticeable differences from human pathogenesis including an absence in cytotoxic T-cell lymphocyte response to HIV infection[164-167] . In addition, many regions consider chimpanzees to be an endangered species which greatly restricts their availability for research. Therefore, scientists turned to alternative models to represent HIV-1 transmission; SIV and SHIV. SIV is the most similar virus to HIV. Therefore many of the aspects of SIV infection in simians could provide insight on HIV infection in human.

Over 40 species of African monkeys are endemically infected with a species specific SIV [168]. Of these speces, the sooty mangabey (SMs) and the African green monkey (AGM) are considered to be natural SIV hosts while other species such as the rhesus macaque (RM) are not natural hosts. Natural SIV hosts do not typically develop AIDS despite high viral replication [169-174]. These animal models do not develop AIDS, have high viral replication, maintain healthy levels of peripheral $CD4^+$ T-cells, do not have mucosal immune dysfunction, do not develop chronic immune activation, and do not often pass SIV infection by MTCT [169-174]. In contrast, non-natural hosts such as the rhesus macaque (RM) respond to SIV infection similar to

the way how humans respond to HIV infection. SIV-infected RMs will progress to AIDS, lose peripheral CD4^{+} T-cells, develop mucosal immune dysfunction, have increased microbial translocation, develop chronic immune activation, and can readily pass SIV through MTCT replication [169-174]. These dramatic differences in how host species respond to SIV infection has led to great insights into understanding the human response to HIV infection [168]. The mechanisms by which natural hosts limit the effects of SIV infection may be useful for developing novel HIV therapeutic strategies.

To further enhance the usefulness of the non-human primate models, investigators have developed viral strains that mix the genes of SIV with HIV to create chimera SHIVs. Multiple SHIVs have been created with varying mixtures of SIV and HIV genes. In general, SHIVs must contain a SIV *env* gene to enable the virus to infect simian cells. SHIVs have been used to evaluate the species specificity of viral proteins such as vif and vpu.

Humanized Mouse Models

Twenty years after the discovery of HIV, a novel model became available for transmission studies: the humanized mouse. Initially, this model was limited to genetically immune deficient mice (SCID and Bg/Nu/XID) who received a human peripheral blood transplantation [175-177]. Then, humanized mice were improved by the availability of different genetic backgrounds and human cell/tissue engraftment techniques. The following work was done utilizing the bone marrow/liver/thymus (BLT) humanized mouse model. This humanized mouse model is made with NOD/SCID IL-2 receptor gamma chain knockout mice that have been irradiated, implanted with human thymus and liver tissue under the kidney capsule, and received

a bone marrow transplant with autologous hematopoietic stem cells. BLT mice harbor a *de novo* generated human immune system distributed throughout each animal [178-200].

BLT mice recapitulate nearly all of the features of HIV transmission and infection. BLT mice are capable of mucosal transmission of HV via vaginal, rectal, or oral routes and sustain HIV infection for the duration of their lifespan [201-205]. Studies utilizing these animals have demonstrated the presence of a viral reservoir much like that of human HIV patients [179]. HIV-infected mice respond to ART and can develop drug resistant HIV infections when ART is given in suboptimal doses [179, 206]. Therefore, BLT mice provide an excellent small animal model for testing the efficacy of HIV transmission prevention strategies where enough animals can be used to generate statistically meaningful results.

Previously, BLT mice were used to test the protective effect of systemically administered antiretroviral drugs to prevent HIV transmission after rectal or intravenous exposure [184]. Briefly, BLT humanized mice were administered daily FTC/TFV for seven consecutive days. On the third day, mice were exposed rectally or intravenously to HIV-1. Mice receiving PrEP were completely protected from rectal transmission of HIV and had only one breakthrough infection (1 of 8 mice) when exposed intravenously [184]. The BLT mouse model has also been used to recapitulate the human clinical trial CAPRISA004. In this clinical trial, participants were instructed to apply a 1% tenofovir gel vaginally before and after coitus [40, 93]. The human clinical trial found topically applied tenofovir to be protective against vaginal HIV. The same topical tenofovir PrEP was found to be protective utilizing the humanized BLT model [180]. Within the same humanized mouse study, six additional topical microbicide candidates were successfully evaluated [180] . Of the six topical microbicides, four were found protecteive 1) C52L, a C-peptide fusion inhibitor at 500uM concentration, 2) topical combination of 16.5uM

TDF and 28uM FTC , 3) C5A, a membrane-disrupting amphipathic peptide inhibitor at 200uM concentration, and 4) PIE 12-Trimer, a trimeric D-peptide fusion inhibitor at 100uM concentration. Of the six topical microbicides tested, a thioester compound (TC247) at 500uM concentration one was found to only offer partial protection (50%) and a small molecule Rac inhibitor (NSC23766,Calbiochem) at 10mM concentration was found to have no protection at all.

Humanized mice have also been useful for evaluating effective ART for HIV treatment. In one study, BLT mice were infected with HIV and treated with daily injections of ART (200mg/kg body weight FTC, 208mg/kg TDF, and 80mg/kg RAL) resulting in viral load suppression to undetectable levels within two weeks [207]. Interruption o f ART resulted in viral rebound [208].

In summary, humanized mice provide a reliable small animal model to evaluate novel HIV transmission interventions. They are susceptible to HIV transmission through vaginal, rectal, wound/intravenous, and/or oral exposure. Previous studies with humanized mice have shown them capable of evaluating both systemic and topical PrEP strategies.

SIGNIFICANCE AND OBJECTIVES

The only way to stem the HIV/AIDS epidemic is to prevent viral transmission to uninfected individuals. In the past, behavior modification strategies, HIV testing, encouraging condom usage, and discouraging sharing needles have all helped to prevent the spread of HIV along with other pathogens. Recommendations such as Option B or Option B+ have greatly

reduced mother-to-child transmission rates in regions with access to antiretroviral drugs. To date, sexual transmission of HIV is the greatest driver of the HIV/AIDS epidemic. Consistent condom usage and male circumcision have both proven to be effective at reducing HIV transmission but require the penetrating partner to consent and accept these practices.

Topically applied interventions or microbicides, on the other hand, may have higher acceptability due to their similarity to already available sexual products/lubricants, their possible availability over-the-counter, and local administration of product may not result in as many side effects or complications. Therefore, the main objective of my dissertation is to explore factors affecting mucosal transmission of HIV and evaluate forms of HIV prevention that are applied topically.

Topically applied PrEP/microbicides designed to prevent HIV transmission have been and are currently being tested for efficacy is multiple clinical trials. Among these interventions, antiretroviral drugs applied topically for PrEP is the furthest advanced through clinical trials. Therefore, part of my dissertation work evaluated the efficacy of topically applied antiretroviral drugs from two different drug classes to prevent HIV transmission. The use of antiretroviral drugs for prevention as well as treatment has raised concerns about transmitted drug resistant HIV. To address this I demonstrated how drug resistant HIV may be transmitted albeit inefficiently. Topically applied interventions that are not antiretroviral based are also under investigation. Among these are interventions aimed at affecting the commensal flora at the site of exposure to prevent mucosal HIV transmission. To evaluate the effect of commensal flora on HIV transmission, I developed a humanized mouse model with a human associated microbiome. Utilizing these mice, it was determined that commensal microbiota composition does affect HIV transmission.

Overall, by using humanized mice as an experimental platform, we expanded our knowledge of mucosal HIV transmission prevention strategies. Knowledge gained from this work supports the development of tenofovir and/or Maraviroc based PrEP for human use, reduces some of the concern of transmitted tenofovir resistant HIV, and provides powerful empirical evidence supporting microbial involvement in HIV transmission.

CHAPTER II – RECTAL TRANSMISSION OF TRANSMITTED/FOUNDER HIV-1 IS EFFICIENTLY PREVENTED BY TOPICAL 1% TENOFOVIR IN BLT HUMANIZED MICE[1]

AUTHOR SUMMARY

Rectal microbicides are being developed to prevent new HIV infections in both men and women. We focused our in vivo preclinical efficacy study on rectally-applied tenofovir. BLT humanized mice (n = 43) were rectally inoculated with either the primary isolate HIV-1JRCSF or the MSM-derived transmitted/founder (T/F) virus HIV-1THRO within 30 minutes following treatment with topical 1% tenofovir or vehicle. Under our experimental conditions, in the absence of drug treatment we observed 50% and 60% rectal transmission by HIV-1JRCSF and HIV-1THRO, respectively. Topical tenofovir reduced rectal transmission to 8% (1/12; log rank p = 0.03) for HIV-1JRCSF and 0% (0/6; log rank p = 0.02) for HIV-1THRO. This is the first demonstration that a human T/F virus rectally infects humanized mice and that its transmission can be efficiently blocked by rectally applied 1% tenofovir. These results obtained in BLT mice, along with recent ex vivo, Phase 1 trial and non-human primate reports, provide supportive evidence to promote the development of tenofovir-based rectal microbicides.

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Author contributions are as follows: conceived and designed the experiments, MC MS; IM, VG performed the experiments, MC ; analyzed the data, MC; wrote the paper, MC, PD, MS, IM, VG

INTRODUCTION

Efficacious biomedical HIV prevention interventions could dramatically reduce the number of new HIV infections globally [209-216]. Microbicides (also referred to as topical pre-exposure prophylaxis [topical PrEP]) represent one of several classes (e.g. oral PrEP, treatment-as-prevention) of such interventions currently being developed [34, 217-222]. There are multiple reasons why microbicides are attractive as tools for HIV prevention: (i) local administration of an antiretroviral gel at the site of exposure will result in higher drug levels at the intended anatomical location than can be achieved using oral PrEP [28, 31-33] while reducing the likelihood of experiencing systemic dosing-associated toxicities [33, 34]; (ii) the reduced toxicity associated with topical microbicides is expected to increase adherence [35]; (iii) microbicides are user controlled [28]; (iv) microbicides are predicted to be cost-effective [29, 30]; (v) topical microbicides can be developed with combinations of viral inhibitors [36]; (vi) an ideal microbicide would be safe and effective in both rectal and vaginal compartments [37-39]; and (vii) antiviral microbicides may also protect against viruses other than HIV (e.g. herpes simplex) [40, 41].

All microbicide efficacy clinical trials to date have tested the prevention of vaginal HIV transmission [35, 56, 75, 78, 213, 217, 223-227]. However, an important driver of the epidemic in both men and women is HIV transmission resulting from anal intercourse [228-235] such that rectal microbicide development is also required [35, 53, 94, 236-238]. Proof of concept that administration of an antiretroviral gel rectally can prevent transmission of SIV/SHIV has been demonstrated for tenofovir [239] and MIV-150 [240]. Tenofovir, UC781, and nonoxynol-9 have been tested for safety and acceptability in Phase 1 rectal microbicide clinical trials [32, 33, 55,

241]. Of these three candidates, only tenofovir 1% gel has advanced to Phase 2 rectal testing and could be considered for Phase 3 efficacy trials in the future [35]. Therefore, our *in vivo* preclinical efficacy study in bone marrow-liver-thymus (BLT) humanized mice was designed to determine the efficacy of topical tenofovir for the prevention of rectal HIV-1 transmission.

BLT mice are the experimental platform of choice for this study for several reasons. For example, BLT mice harbor a *de novo* generated human immune system distributed throughout each animal [178-200]. In the context of this study, an important characteristic of BLT mice is their susceptibility to rectal HIV-1 transmission [184, 187] due to the presence of human $CD4^+$ T cells, macrophages and dendritic cells found throughout BLT mouse intestines, including the rectum [178, 187], previously both topical [180] and systemic [183, 184] HIV prevention interventions have been extensively tested in BLT mice for their ability to block vaginal transmission of HIV-1. The results obtained from these studies were highly predictive of the clinical trial outcomes [180, 183, 184, 217, 221, 242].

An important and novel aspect of this study is the use of a MSM-derived transmitted/founder (T/F) virus [243]. Typically only one or a few virions (defined as the T/F viruses) are responsible for a mucosal transmission event in humans making T/F viruses physiological relevant for *in vivo* efficacy studies of HIV prevention interventions [244, 245]. BLT mice were treated rectally with topical 1% tenofovir and then rectally inoculated with HIV-1_{JRCSF}, a well characterized low passage primary isolate, or the T/F virus HIV-1_{THRO}. We found that rectal transmission of both viruses was efficiently prevented by topical tenofovir.

RESULTS

Human PBMC reconstitution of BLT mouse pre-exposure.

This study was designed to determine the *in vivo* efficacy of topical tenofovir for the prevention of rectal HIV-1 transmission. Prior to HIV-1 exposure of the BLT mice, their peripheral blood was characterized by flow cytometry to confirm reconstitution with human cells. All BLT mice used herein (n=43) had high peripheral blood reconstitution levels of human lymphoid ($CD45^+$) cells (67% mean ± 17 SD) and human $CD4^+$ T cells (80% mean ± 6 SD) (Summarized in Tables 1 and 2).

Topical tenofovir prevents rectal HIV-1JRCSF transmission.

A total of 29 mice were exposed to HIV-1_{JRCSF}, a CCR5-tropic virus that has been well characterized for its mucosal infection of BLT mice [180, 181, 183, 184, 187, 199, 200]. Seventeen mice received vehicle and 12 mice received topical tenofovir (Figure 2; Table 1). Following viral exposure, peripheral blood from the BLT mice was sampled weekly for the presence of HIV-1 RNA (Figure 1). Eight of the 17 mice in the control arm of the experiment were infected as determined by the presence of viral RNA in plasma (Figure 2A). In contrast, 11 of 12 topical tenofovir treated mice were consistently negative for the presence of plasma viral RNA (Figure 2A). One tenofovir treated mouse was found to have a ‘breakthrough’ infection with readily detectable plasma viral RNA (Figure 2A). No tenofovir resistant mutations from this breakthrough virus were identified when the entire reverse transcriptase gene was sequenced. Over the course of this experiment, we also monitored the levels of $CD4^+$ T cells in peripheral

blood. The breakthrough infection mouse and the infected vehicle control mice had peripheral blood $CD4^+$ T cell levels similar to the HIV-1 negative mice (Figure 2B).

Prior to defining topical tenofovir treated BLT mice as protected from rectal HIV-1 transmission, we tested tissues harvested from these mice for the presence of cell-associated HIV-1 DNA. All mice without plasma viral RNA were also found to be negative for viral DNA in all tissues evaluated (e.g. bone marrow, spleen, human thymic organoid and lymph nodes) confirming the lack of HIV-1 transmission in these animals (Figure 2C; Table 1). The HIV status and time to plasma viremia were then combined to generate a Kaplan-Meier plot of the protection from rectal HIV transmission provided by topical tenofovir (Figure 3). Log rank analysis (p=0.03) confirmed that topical tenofovir prevents rectal HIV-1_{JRCSF} transmission in BLT mice.

Rectal transmission of transmitted/founder HIV-1_{THRO} is prevented by topical tenofovir.

HIV-1_{THRO} is a CCR5-topic, MSM-derived T/F virus [243]. A total of 14 BLT mice were exposed rectally to HIV-1_{THRO} (Figure 4). Eight mice received vehicle and six mice received tenofovir. Five of the mice receiving vehicle were infected as determined by the presence of plasma virus RNA (Figure 4A). In contrast, none of the tenofovir treated BLT mice (0/6) exposed rectally to HIV-1_{THRO} exhibited plasma viremia (Figure 4A). In addition to plasma viremia, we also monitored the levels of human $CD4^+$ T cells in the peripheral blood of all the HIV-1_{THRO} exposed mice. The levels of human $CD4^+$ T cells in the infected mice did not change throughout the course of infection (Figure 4B).

To confirm the lack of HIV-1 infection of the tenofovir treated mice we used real time PCR to determine the presence of cell-associated HIV-1 DNA in tissues obtained from these

mice. None of the mice treated with tenofovir had detectable levels of viral DNA in any of the tissues examined (Figure 4C; Table 2). In contrast, the presence of viral DNA in tissues from infected animals was readily confirmed (Figure 4C; Table 2). Log rank analysis of these results presented in a Kaplan-Meier plot (Figure 5) revealed that topical tenofovir administered prior to exposure to BLT mice prevents rectal transmission of the physiologically relevant T/F virus, HIV-1_{THRO} ($p=0.02$).

DISCUSSION

Mucosal infection after sexual intercourse is the most common route of HIV-1 transmission worldwide which makes the cervicovaginal and rectal mucosa the two most important anatomical sites for viral exposure [246]. Receptive anal intercourse has the highest risk of HIV-1 infection and accounts for most new infections in the US [247, 248]. Nevertheless, the vast majority of past and ongoing clinical trials for HIV prevention using topical microbicides have focused on preventing vaginal HIV-1 acquisition [35, 56, 75, 78, 213, 217, 223-227]. The formulation of tenofovir 1% gel used in the RMP-02/MTN-006 Phase 1 rectal safety study was the same formulation used vaginally in the CAPRISA 004 trial [33, 217]. Unfortunately, there was a significant increase in gastrointestinal adverse events seen in the RMP-02/MTN-006 study, possibly due to the hyperosmolar nature of the gel [33, 35]. We therefore elected to evaluate the efficacy of tenofovir directly, in the absence of any type of gel, to make a clear determination of the potential efficacy of tenofovir for the prevention of rectal HIV transmission. Our study supports the choice of tenofovir as an appropriate active

pharmaceutical ingredient around which a specifically engineered microbicide can be designed for rectal [32, 33, 35] or dual compartment use [38, 39].

Our goal was to evaluate the *in vivo* efficacy of a viable candidate for inclusion into a rectal microbicide to prevent HIV-1 acquisition. We focused on rectal HIV transmission because this route of virus spread continues to be a major contributor to the number of men and women becoming infected with HIV [228-234]. We chose a topical intervention because of the many potential benefits associated with this drug delivery route [28-30, 33-41]. BLT mice were chosen as the experimental platform for this evaluation because previous studies have shown that FDA approved drugs prevent mucosal HIV transmission of the human primary virus isolate HIV-1_{JRCSF} in this model [180, 183, 184]. Here when BLT mice were pretreated with topical tenofovir (or vehicle) and then rectally exposed to HIV-1_{JRCSF}, we found that topical tenofovir efficiently prevents rectal transmission of HIV-1_{JRCSF} (Figures 2 and 3; Table 1).

To extend and expand on this observation we also evaluated the protective effect of tenofovir using a second virus, HIV-1_{THRO}. HIV-1_{THRO} is a MSM-derived T/F virus and therefore its evaluation in the context of rectal transmission is of significant relevance [243]. T/F viruses represent the one or few founder viruses that undergo amplification in local T cells and subsequent systemic dissemination after mucosal exposure [243-245, 249]. These T/F viruses use CCR5 as a coreceptor for entry and replicate poorly in monocyte/ macrophages relative to T cells [243]. Despite their intrinsic relevance, T/F viruses have not been previously used for *in vivo* transmission studies in animal models. We found that HIV-1_{THRO} transmits rectally in BLT mice and that its transmission can be efficiently prevented by pretreatment with rectally applied tenofovir (Figures 4 and 5; Table 2).

Analysis of the data from two HIV-1 isolates indicates that 1 of 18 BLT mice became infected despite treatment with topical 1% tenofovir prior to rectal HIV-1 exposure, while 13 of 25 vehicle treated BLT mice became infected (p=0.002 Fisher's exact test) (Tables 1 and 2). In an *in vivo* study using non-human primates (NHP), 2 of 6 macaques became infected despite treatment with topical 1% tenofovir 15 minutes prior to rectal SIV exposure, while 3 of 4 vehicle treated macaques became infected [239]. The conclusion reached by the authors of the macaque study and our conclusion of the study presented here are that topical tenofovir can inhibit rectal transmission of SIV [239], primary HIV-1 (Figure 3) and T/F HIV-1 (Figure 5).

Topical microbicides are of significant interest in HIV prevention because they achieve high local drug concentrations capable of preventing HIV transmission with reduced risk for toxicity [28, 33, 34]. The *in vivo* preclinical efficacy data presented here together with previous data from NHP [239] show that topical tenofovir can efficiently block rectal transmission. The incorporation of a physiologically relevant T/F HIV-1 into this study of rectal HIV prevention increases its translational value. The results presented here show the importance of animal models for the evaluation of HIV-1 prevention strategies and demonstrate the potential for efficacy of tenofovir-based rectal microbicides in humans. Future studies will leverage the results from this work and the BLT model to perform dose-ranging tenofovir studies, evaluate rectal-specific gel formulations containing tenofovir and evaluate other topical rectal microbicide agents for efficacy.

MATERIALS AND METHODS

Preparation of BLT mice and characterization of human reconstitution.

BLT mice were prepared essentially as previously described [178-185, 187, 200]. Briefly, thy/liv implanted [250] and preconditioned NOD/SCID-gamma chain null (NSG) mice (Jackson Laboratories, Bar Harbor, ME) were transplanted with autologous human fetal liver $CD34^+$ cells (Advanced Bioscience Resources, Alameda, CA) and monitored for human reconstitution in peripheral blood by flow cytometry [183, 185, 187]. Mice were maintained at the University of North Carolina at Chapel Hill Division of Laboratory Animal Medicine in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Topical application of tenofovir and rectal exposure of BLT mice to HIV-1.

Stocks of HIV-1_{JRCSF} [251] and HIV-1_{THRO} [243] were prepared and titered as we have previously described [181, 252]. Mice were exposed rectally using 0.6 μg p24 of HIV-1_{JRCSF} ($4x10^6$ TCIU, tissue culture infectious units) and 0.7 μg p24 of HIV-1_{THRO} ($5x10^6$ TCIU). Topical tenofovir consisted of 1% tenofovir (PMPA; 9-(2-phosphonyl-methoxypropyly)-adenine) in PBS. The vehicle (placebo) control was PBS.

The exposure timeline (Figure 1) consisted of rectal application of vehicle or of 1% tenofovir less than 30 minutes prior to rectal application of virus. Rectal exposures with HIV-1_{JRCSF} and HIV-1_{THRO} were performed essentially as previously described [184, 187] except that all the mucosal exposures were carried out atraumatically and without simulated rectal

intercourse [253]. All rectal applications of vehicle or inhibitor as well as virus were performed while mice were anesthetized [184, 187]. After viral exposure, mice were returned to their housing to recover and were then monitored longitudinally for evidence of HIV-1 infection as indicated below.

Analysis of HIV-1 infection of BLT mice.

Infection of BLT mice with HIV-1 was monitored at the indicated time intervals in peripheral blood by determining plasma levels of viral RNA using real time PCR (limit of detection 750 copies/ml) [179, 180] and by monitoring $CD4^+$ T cell percentages by flow cytometry [183, 184]. At necropsy, tissues were harvested and mononuclear cells isolated as previously described [178, 180, 183, 185, 187]. Mononuclear cells were washed, enumerated and tested using real time PCR for the presence of HIV-1 DNA (limit of detection 10 copies) [180, 181, 183, 184].

Sequence analysis was performed on plasma RNA samples in the sole case of breakthrough infection of a tenofovir-treated, HIV-1_{JRCSF}-exposed BLT mouse. The entire reverse transcriptase gene from plasma HIV-1 RNA amplification products was sequenced. No resistance mutations in reverse transcriptase were present [103, 115, 116, 254].

Statistics.

All statistical analyses (alpha level: 0.05) were performed using Prism v. 5 (Graph Pad Software). Kaplan-Meier plots indicate the percentage of animals that are HIV-1 positive in the

peripheral blood at each time point analyzed. Power analysis calculation for experimental group sample sizes were determined as previously described [255, 256]. Briefly, we assumed 50 and 65% variance in transmission between our experimental groups for HIV-1_{JRCSF} and HIV-1_{THRO}, respectively. In the case of each viral isolate, the chosen sample sizes were determined to have 90% power to detect statistically significant differences via log rank test analysis in the treatment arm versus the vehicle arm.

Table 1. BLT mice used to test the efficacy of topical tenofovir to prevent rectal **HIV-1$_{\mathbf{JRCSF}}$** transmission.*

	Mouse	% human CD45$^+$ in PB at exposure	% hCD45$^+$ hCD3$^+$ hCD4$^+$ in PB at exposure	Tissue Cell associated viral DNA^	HIV Status
Topical Tenofovir	J01	78	87	B, S, O, LN	Neg
	J02	69	86	B, S, O, LN,	Neg
	J03	67	79	B, S, O, LN	Neg
	J04	39	83	B, S, O, LN	Neg
	J05	78	67	**B,** S, **O, LN**	**Pos**
	J06	54	84	B, O, LN	Neg
	J07	69	71	B, S, LN	Neg
	J08	73	68	BM, S, O, LN	Neg
	J09	86	69	ND	Neg
	J10	65	88	BM, S, O, LN	Neg
	J11	79	86	B, S, O	Neg
	J12	80	86	BM, S, O, LN	Neg
Mean (+/- SD)		70% (+/-13)	80% (+/-8)		
Vehicle	J13	73	88	**B, S, O, LN**	**Pos**
	J14	52	79	ND	Neg
	J15	32	85	ND	Neg
	J16	84	73	**B,** S, **O,** LN,	**Pos**
	J17	68	86	ND	**Pos**
	J18	56	85	ND	Neg
	J19	31	79	B, S	**Pos**
	J20	83	72	B, O, S,	Neg
	J21	61	71	**S, O, LN,**	**Pos**
	J22	71	83	ND	Neg
	J23	79	88	B, S, O, LN	Neg
	J24	62	84	B, S, O, LN	Neg
	J25	72	84	ND	Neg
	J26	60	80	**S, O, LN**	**Pos**
	J27	75	82	B, S, O, LN	Neg
	J28	76	88	**B, S, O, LN**	**Pos**
	J29	77	87	ND	**Pos**
Mean (+/- SD)		65% (+/-16)	82% (+/-6)		

*The data shown in the table includes analyses performed on both infected and uninfected mice with the text in bold used to highlight that HIV-1 was found in the indicated tissues.

^Abbreviations: B – bone marrow; LN –lymph nodes; ND - not done; Neg – negative; O – thymic organoid; PB – peripheral blood;; Pos – positive; and S – spleen.

Table 2. BLT mice used to test the efficacy of topical tenofovir to prevent rectal **HIV-1$_{THRO}$** transmission.*

	Mouse	% human CD45+ in PB at exposure	% hCD45+ hCD3+ hCD4+ in PB at exposure	Tissue Cell associated viral DNA^	HIV Status
Topical Tenofovir	T01	56	83	B, S, O, LN	Neg
	T02	81	81	B, S, O, LN	Neg
	T03	82	77	B, S, O, LN	Neg
	T04	87	82	B, S, O, LN	Neg
	T05	24	80	B, S, O, LN	Neg
	T06	29	80	B, S, O, LN	Neg
Mean (+/- SD)		60% (+/-28)	81% (+/-2)		
Vehicle	T07	61	82	**B, S, O,**	**Pos**
	T08	85	81	B, S, O, LN	Neg
	T09	70	76	**B, S, O**	**Pos**
	T10	86	78	**B, S, O**	**Pos**
	T11	42	83	**B, S, O**	**Pos**
	T12	56	77	ND	Neg
	T13	83	75	B, S, O,LN	Neg
	T14	73	78	ND	**Pos**
Mean (+/- SD)		70% (+/-16)	79% (+/-3)		

*The data shown in the table includes analyses performed on both infected and uninfected mice with the text in bold used to highlight that HIV-1 was found in the indicated tissues.

^Abbreviations: B – bone marrow; LN –lymph nodes; ND - not done; Neg – negative; O – thymic organoid; PB – peripheral blood;; Pos – positive; and S – spleen.

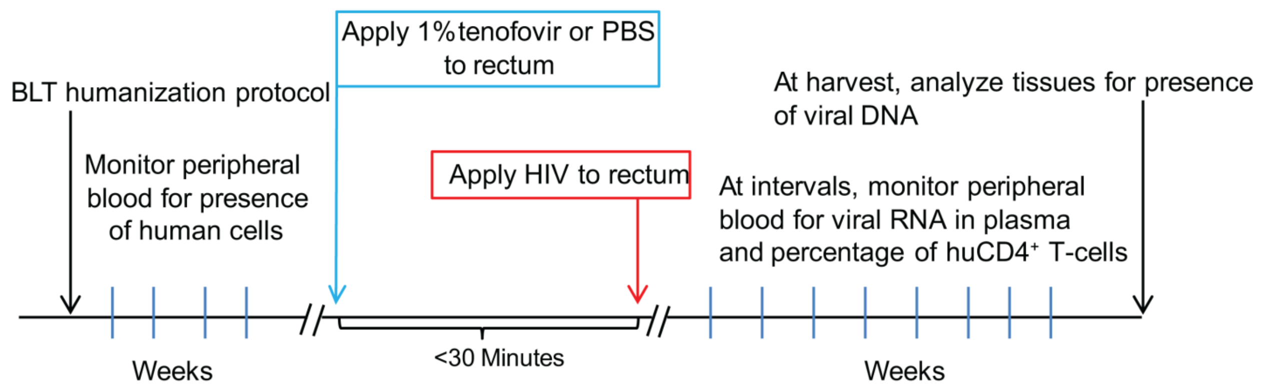

Figure 1: Experimental design and timeline

BLT mice were utilized to determine the efficacy of topically applied tenofovir to prevent rectal HIV-1 transmission. Rectal HIV-1 exposures were performed within 30 minutes following rectal application of 1% tenofovir. Plasma viral load and real time PCR amplification of tissue associated viral DNA were used as HIV-1 detection strategies to determine whether peripheral blood samples collected at the indicated times and tissues collected at harvest contained HIV-1.

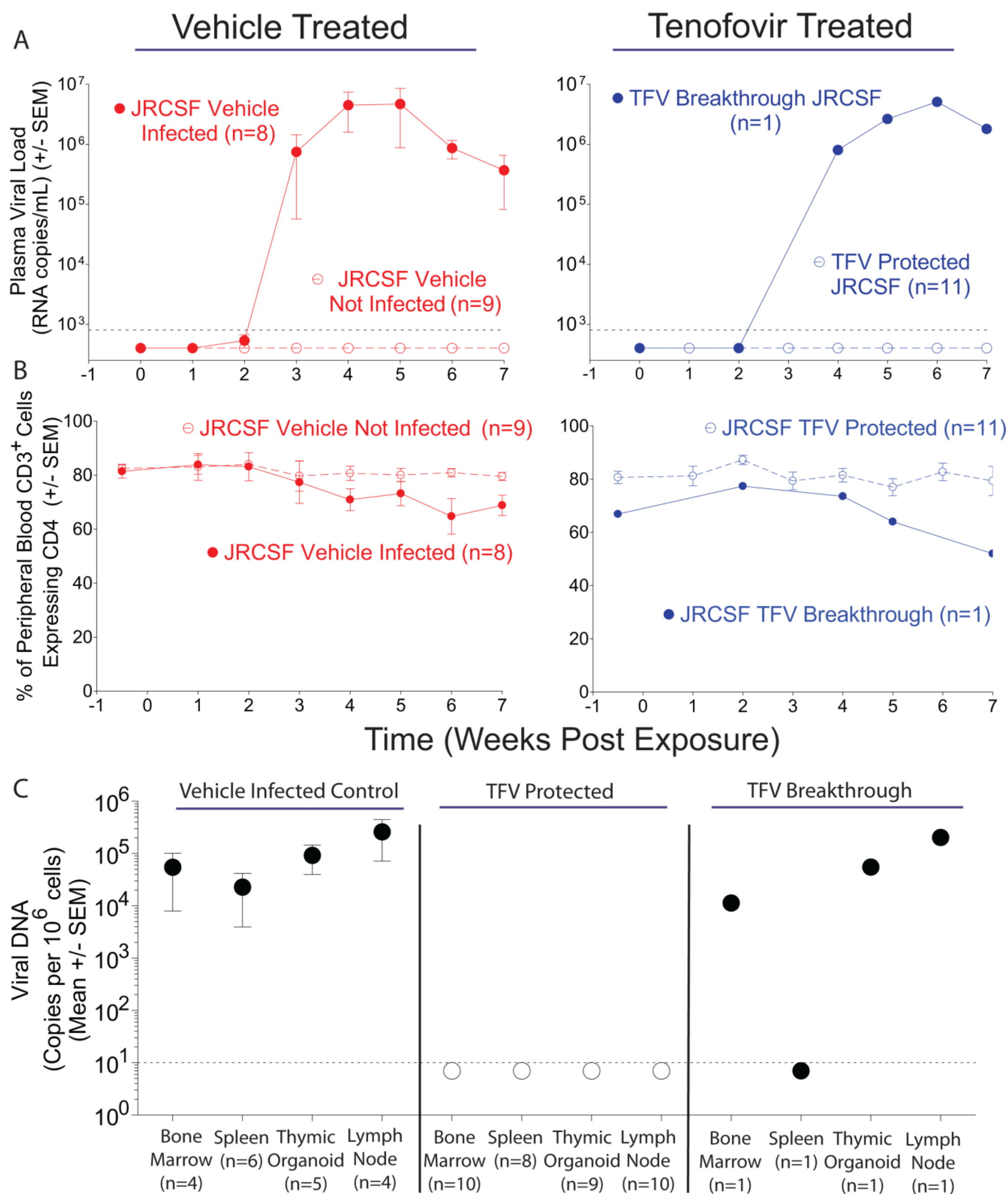

Figure 2: Analysis of peripheral blood and tissues for the presence of HIV-1$_{JRCSF}$ after rectal exposure in the presence or absence of topical tenofovir

(A-B) Longitudinal analyses of peripheral blood plasma viral RNA (A) and the percentage of peripheral blood CD3$^+$ T cells also expressing CD4 (B) are presented for vehicle (left) and topical tenofovir (right) -treated BLT mice exposed rectally to HIV-1$_{JRCSF}$. (C) Real-time PCR analysis of tissues for presence or absence of HIV-1 DNA. Thin dashed lines represent the limit of detection for the respective assays. Error bars indicate standard error of the mean. Open symbols are used to depict data from HIV negative mice and closed symbols are used to depict data from HIV positive mice.

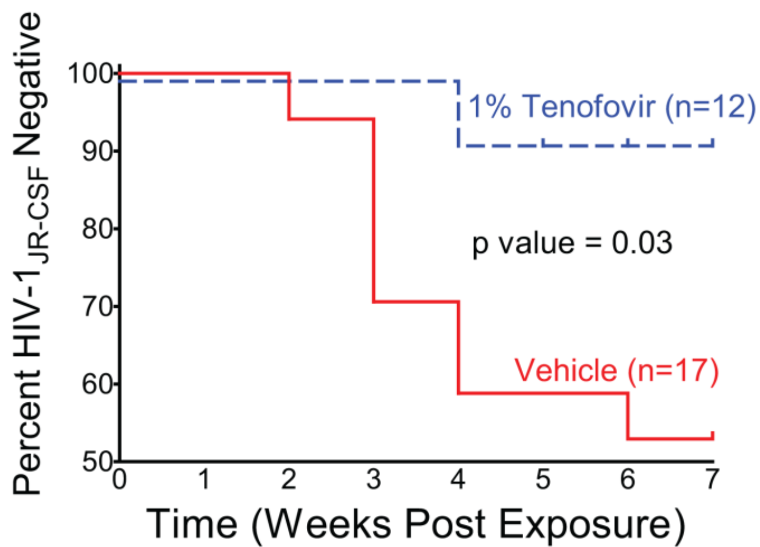

Figure 3: Topical tenofovir prevents rectal HIV-1_{JRCSF} transmission in BLT mice.

Kaplan-Meier plot indicates the time to detectionof viral RNA or DNA following rectal HIV-1_{JRCSF} exposure in BLT mice pretreated with either vehicle or topical tenofovir. Log-rank (Mantel Cox) analysis reveals a statistically significant difference in rectal HIV-1_{JRCSF} transmission between the vehicle and topical tenofovir arms.

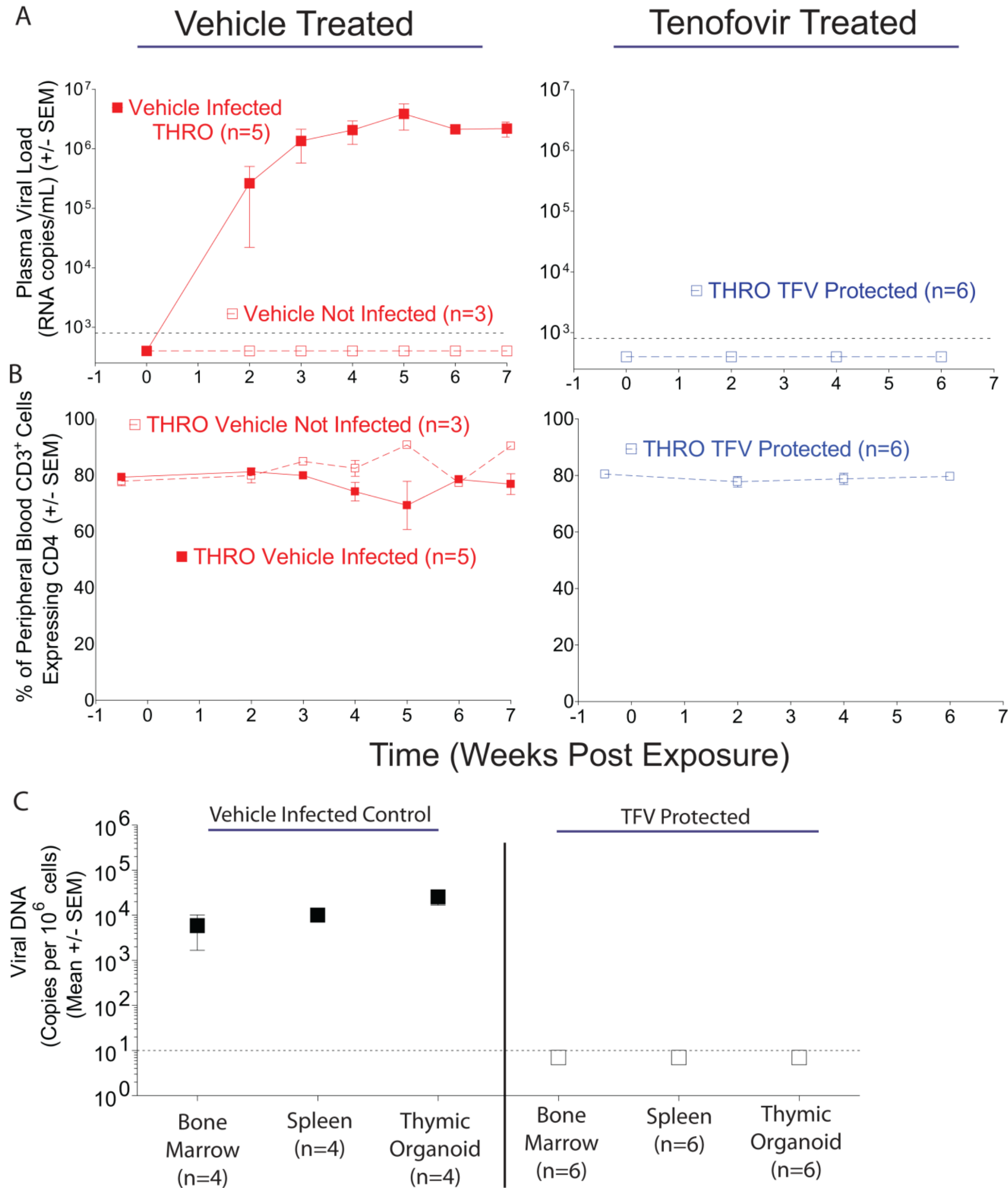

Figure 4: Analysis of peripheral blood and tissues for the presence of HIV-1$_{THRO}$ after rectal exposure in the presence or absence of topical tenofovir

(A-B) Longitudinal analyses of peripheral blood plasma viral RNA (A) and the percentage of peripheral blood CD3$^+$ T cells also expressing CD4 (B) are presented for vehicle (left) and topical tenofovir (right) -treated BLT mice exposed rectally to HIV-1$_{THRO}$. (C) Real-time PCR analysis of tissues for presence or absence of HIV-1 DNA. Thin dashed lines represent the limit of detection for the respective assays. Error bars indicate standard error of the mean. Open symbols are used to depict data from HIV negative mice and closed symbols are used to depict data from HIV positive mice.

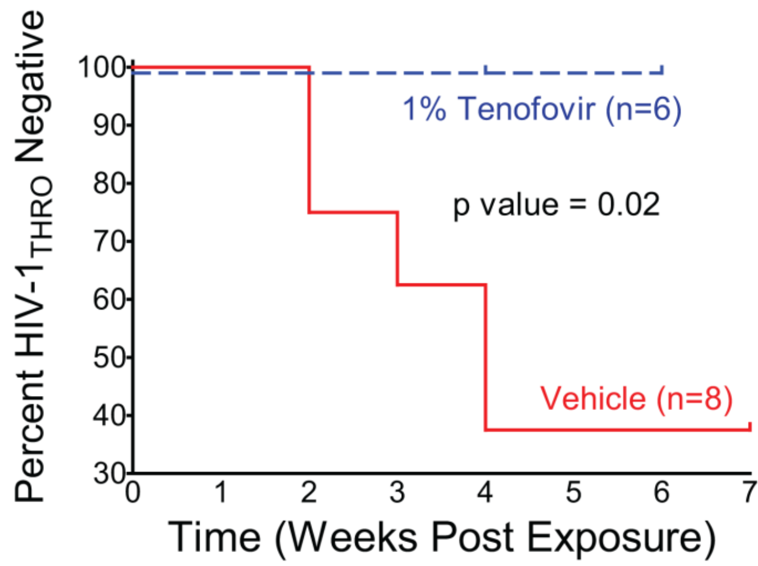

Figure 5: Topical tenofovir prevents rectal transmission of HIV-1$_{THRO}$, a T/F virus, in BLT mice

Kaplan-Meier plot indicates the time to detection of viral RNA or DNA following rectal HIV-1$_{THRO}$ exposure in BLT mice pretreated with either vehicle or topical tenofovir. Log-rank (Mantel Cox) analysis reveals a statistically significant difference in rectal HIV-1$_{THRO}$ transmission between the vehicle and topical tenofovir arms.

CHAPTER III. INEFFICIENT VAGINAL TRANSMISSION OF TENOFOVIR RESISTANT HIV-1 [2]

AUTHOR SUMMARY

Transmission of drug resistant HIV has been postulated to be a threat to current first line antiretroviral therapy (ART) regimes and the efficacy of several antiretroviral based pre-exposure prophylaxis (PrEP) strategies being tested. Here we evaluated the effect of the common tenofovir (TFV) resistance mutation, K65R on vaginal HIV transmission. Our results demonstrate that despite no overt loss of overall replication competence *in vivo*, this mutation results in significantly reduced mucosal transmission. When transmitted the mutant virus eventually reverts to wild type in the absence of drug therapy.

INTRODUCTION

In absence of a cure or vaccine, and despite valuable efforts toward better HIV education including safe sex practices, the HIV epidemic continues to grow at a faster pace than the current availability of antiretroviral therapy (ART). For every two people who begin ART, five are newly infected [2]. Of the people infected, only 47% have access to ART in low and middle income countries [3]. There is a great need to prevent transmission of HIV. To address this need,

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[2] This chapter previously appeared as an article in the journal of . The original citation is a follows: M.L. Chateau, P.W. Denton, M.D. Swanson, I. McGowan, and J.V. Garcia. Inefficient Vaginal transmission of tenofovir resistant HIV-1. PLoS One. 2013;8(3):e60024.

Author contributions are as follows: conceived and designed the experiments, MC MS; performed the experiments, MC, MS ; analyzed the data, MC; wrote the paper, MC, PD, MS, IM, VG

extensive efforts are being made to develop and implement effective pre-exposure prophylaxis (PrEP) approaches. So far, the greatest progress has been made using antiretroviral drug based treatment as prevention and PrEP [14, 93]. When the patient has a positive diagnosis and access to ART, then early treatment is highly effective at preventing transmission of HIV to uninfected partners [14]. Unfortunately, a significant number of HIV+ individuals do not know their infection status, especially during acute infection when transmission potential is highest [248]. Most current PrEP clinical trials are investigating the use of antiretroviral drugs either singularly or as a two drug combination for systemic or topical use [21, 22, 24, 26, 93]. This raises an important concern with the dual use of antiretroviral drugs for both treatment and prevention: the consequences of the development and transmission of drug resistant HIV.

HIV-1 develops resistance to virtually all drugs currently available for treatment [103, 104]. For this reason, current ART therapies consist of a cocktail of multiple drugs with different classes of action to prevent or at least postpone the development of drug resistant HIV within the patient's lifespan. Drug-resistant viruses can be transmitted [121-124] . During new infections certain mutations like M184V are rarely detected by routine genotyping but significantly higher proportions can be detected using more specific methodology [121-123]. The inherent ability of replicating HIV to revert to a drug sensitive genotype in the absence of drug pressure makes it difficult to study in patients especially if: (1) the time/duration/route of infection are unknown, (2) there is no way to prove ART naïve status, and (3) the HIV sequence in the infecting partner is unknown. Despite these difficulties, genotypic analysis of ART naïve patients has provided evidence that drug resistant HIV-1 is being transmitted and can result in treatment failure [125-

131]. Given that animal studies are the best option to overcome the inherent limitations of human studies [257], we utilized humanized mice to investigate *in vivo* transmission of a drug resistant HIV-1.

Currently, transmitted drug resistant HIV is very rare, but the novel use of antiretroviral drugs for prevention as well as treatment has generated some concerns. Since tenofovir is the furthest along for development as a topical microbicide and is one of the two drugs that make up the only approved systemic PrEP Truvada, it is logical to look at tenofovir resistant HIV. Given that animal studies are the best option to overcome the inherent limitations of human studies [257], we utilized humanized mice to investigate *in vivo* transmission of a drug resistant HIV-1.

Tenofovir (TFV) is the drug most commonly used in clinical trials evaluating systemic and topical PrEP. Tenofovir disoproxil fumarate, the oral formulation of TFV, is also part of every DHHS recommended first line therapy [258]. For this reason, we chose to study transmission of a tenofovir resistant HIV. The mutation of the lysine at amino acid position 65 in HIV reverse transcriptase to an arginine (K65R) confers resistance to tenofovir as well as other NRTIs. For this reason K65R is on both the WHO and IAS surveillance lists for HIV genotyping [103, 104]. Analysis of crystal structures suggests that the lysine at position 65 normally interacts with the incoming dNTP to form a salt bridge between the gamma phosphate of the dNTP and the epsilon amino group of the lysine [116, 259]. This interaction is lost when arginine is substituted for lysine and therefore the interactions between enzyme and triphospahte bearing molecules (like NRTIs and dNTP) is reduced [116]. The end result is a reduced replication capacity (loss of dNTP interaction) but a reduced susceptibility to tenofovir (loss of NRTI interactions) [116].

There is clinical evidence that HIV containing the K65R mutation can be transmitted after mucosal exposure albeit at lower frequency than other mutations like M184V [121, 122, 125, 131]. To evaluate the role of this single amino acid mutation on mucosal HIV transmission, we introduced the K65R mutation (AAA to AGA) into a proviral clone of HIV-$1_{JR\text{-}CSF}$ [251]. In addition, to differentiate the mutant virus from the parental clone after reversion, a second, silent mutation (TAT to TAC, Tyrosine) was included to act as a molecular marker.

RESULTS

The K65R mutation in viral reverse transcriptase increases resistance to TFV

To confirm a decrease in the susceptibility of the mutant virus to TFV, we determined the *in vitro* IC_{50} for wild type $HIV_{JR\text{-}CSF}$ and the isogenic mutant, $HIV_{JR\text{-}CSF\ K65R}$. The K65R mutation conferred a 4.7 fold increase in the *in vitro* IC_{50} for TFV, which is comparable to the 2 to 4 fold range reduction in susceptibility reported [115, 260] (Figure 6).

$HIV_{JRCSF\ K65R}$ replicates *in vivo* but fitness defects result in reversion to wild type

Previous *in vitro* studies have shown that the K65R mutation reduces the function of viral reverse transcriptase [115, 119]. It is unknown to what extent this defect affects viral replication *in vivo*. To test the *in vivo* replication capacity of $HIV_{JR\text{-}CSF\ K65R}$, humanized mice [261, 262] were inoculated via IP injection $3x10^4$ TCIU and viral load in plasma was monitored over time [180]. Longitudinal analysis of plasma viral load showed no difference in the *in vivo* replication of the K65R and wild type strains (Figure 7) demonstrating the *in vivo* fitness of the mutant virus. Sequence analysis of plasma virus RNA from HIV-$1_{JR\text{-}CSF\ K65R}$ infected mice confirmed the presence of the K65R mutation 2 weeks post infection. However, subsequent time points

showed a population of wild type virus. Sequence analysis indicated that reversion of the K65R mutation was always to the original sequence. It should be noted that the molecular marker, present only in the mutant virus, served to exclude the possibility of contamination with wild type virus.

Having demonstrated the replication capacity of the K65R mutant virus *in vivo*, we next evaluated its capacity to transmit mucosally. For this purpose, we utilized BLT humanized mice [180]. The female reproductive tract of BLT mice is reconstituted with all the cells relevant for HIV transmission including human T cells, monocyte/macrophages and dendritic cells [180, 204]. BLT mice were vaginally exposed once to equal infectious doses of wild type HIV-1_{JR-CSF} or the isogenic K65R mutant virus ($3.5X10^5$ TCIU). Three independent exposures (n=4) were performed on three different dates. The results of these vaginal exposures showed a dramatic decrease in the transmission efficiency of the K65R mutant virus (Figure 8). Specifically, whereas all the mice exposed to the wild type virus were infected (4/4) only 25% of the mice exposed to the mutant virus were infected (3/12). This difference in vaginal HIV transmission was highly statistically significant by log rank analysis (p=0.011, Mantel Cox). These results demonstrate that the K65R mutant is vaginally transmitted at a greatly reduced rate compared to the wild type virus. Interestingly, these results seem at odds with those recently published by Cong et al [257] using SIV. However, these could due to the facts that a different mutation was used and that additional fitness compensatory mutations were introduced into the provirus used by Cong et al [257].

To determine if the transmitted virus contained the K65R mutation, plasma viral RNA was sequenced at different times after exposure. Four weeks post exposure we noted the presence of only mutant virus in one mouse (M1), the presence of only wild type (reverted) virus

in a second mouse (M2), and the presence of both mutant virus and wild type (reverted) virus populations in a third mouse (M3). Longitudinal analysis of the virus found in the plasma of one of the infected mice (M3) showed the presence of both mutant and wild type viruses at weeks 4 and 6 post infection and the presence of wild type virus at all subsequent time points (Table 1). Cervicovaginal lavage (CVL) from this mouse also showed the presence of both wild type and mutant virus 4 weeks post infection. Subsequently only the wild type virus was found in the CVL (Table 1). Analysis of the virus present in the different tissues from two of the infected mice generally reflected what was observed in the periphery. However, in one mouse the mutant virus was found in the plasma but all tissues analyzed contained both the wild type and mutant viruses. Interestingly, analysis of the virus present in tissues 14 weeks post infection showed the wild type virus in all tissues except thymus where both drug resistant and wild type virus were found (Table 1). These results are consistent with the hypothesis of Weinberg et al suggesting that transmitted viruses that contain reversible mutations become archived in lymphocyte reservoirs [123].

DISCUSSION

The topical or systemic use of antiretroviral drugs for the purpose of preventing HIV acquisition has the potential to curtail the spread of AIDS and some PrEP strategies have shown great promise [14, 22, 26, 263, 264]. The fact that tenofovir is a successful first line drug for the treatment of HIV infection has made this compound the drug of choice for most prevention trials [265]. However, this dual use approach is not without risk as there is significant potential to expand the pool of drug resistance in communities utilizing PrEP [15, 264]. Here we test K65R mutated HIV-1 in humanized mice and found that, as in humans, the HIV carrying the K65R

mutation is (1) replication competent (Figure 7); (2) is present in cervicovaginal secretions (Table 3); and (3) reverts to wild type in the absence of drug selection although the mutant virus remains detectable (Table 3). The reversion to wild type in our humanized mouse model replicates what is predicted to occur in human patients [266]. It is important to note, that in the absence fo drug pressure, the K65R mutation reverts to a wildtype/drug sensitive genotype both in the peripheral blood and female reproductive tract. This suggests that despite being initially infected with a tenofovir resistant HIV, an individual who is not taking ART is more likely to transmit a drug sensitive form of HIV.

Patients initially infected with a viron contaiing the K65R mutation may form latently infected cells with this tenofovir resistant version of HIV before the virues reversts to a tenofovir-sensitive genotype. Then, upon initiation of tenofovir based ART, there is a greater chance of re-emergence of the original drug resistant form. Fourtunatly, the K65R mutation is specific to NRTI RT inhibitors so a virus containing only a K65R mutation will remain sensitive to protiease, integrage, and NNRTIs.

Finally, we tested the transmission efficiency of the K65R mutant HIV and found that it could transmit, albeit at a significantly lower efficiency than wild type HIV (Figure 8). Several clinical trials have been done to evaluate the efficacy of tenofovir or tenofovir disoproxil fumarate to prevent HIV transmission. One concern has been that a virus containing a tenofovir resistance mutation such as K65R will be able to transmit despite the use of a TFV/TDF PrEP. Given our results, it seams the benefit of drug resistance is overshadowed by a severe defect in transmission. It is possible that the protective efficacy of a TFV/TDF PrEP is sufficient to prevent transmission of K65R containing HIV. Overall, our results demonstrate that if this tenofovir resistant virus is present in the transmitting partner, there is the potential for the mutant

virus to be transmitted to the uninfected partner with lower efficiency compared to wild type HIV-1.

MATERIALS AND METHODS

Preparation of BLT mice and characterization of human reconstitution.

BLT mice were prepared essentially as previously described [178-185, 187, 200]. Briefly, thy/liv implanted [250] and preconditioned NOD/SCID-gamma chain null (NSG) mice (Jackson Laboratories, Bar Harbor, ME) were transplanted with autologous human fetal liver $CD34^+$ cells (Advanced Bioscience Resources, Alameda, CA) and monitored for human reconstitution in peripheral blood by flow cytometry [183, 185, 187]. Mice were maintained at the University of North Carolina at Chapel Hill Division of Laboratory Animal Medicine in accordance with protocols approved by the Institutional Animal Care and Use Committee.

IP andVaginal exposure of BLT mice to HIV-1.

Stocks of HIV-1_{JRCSF} [251] and HIV-$1_{JRCSF(K65R)}$ were prepared and titered as we have previously described [181, 252]. Mice were exposed intraperitoneally (IP) using 3x10^4 TCIU of HIV-1_{JRCSF} or HIV-$1_{JRCSF(K65R)}$. Mice were exposed vaginally using 3.5x10^5 TCIU of HIV-1_{JRCSF} or HIV-$1_{JRCSF(K65R)}$. After viral exposure, mice were returned to their housing to recover and were then monitored longitudinally for evidence of HIV-1 infection as indicated below.

Analysis of HIV-1 infection of BLT mice.

Infection of BLT mice with HIV-1 was monitored at the indicated time intervals in peripheral blood by determining plasma levels of viral RNA using real time PCR (limit of detection 750 copies/ml) [179, 180] and by monitoring $CD4^+$ T cell percentages by flow cytometry [183, 184]. At necropsy, tissues were harvested and mononuclear cells isolated as previously described [178, 180, 183, 185, 187]. Mononuclear cells were washed, enumerated and tested using real time PCR for the presence of HIV-1 DNA (limit of detection 10 copies) [180, 181, 183, 184].

Sequence analysis was performed on RNA and DNA samples in the cases of successful transmission of HIV-$1_{JRCSF(K65R)}$ exposed BLT mouse. The entire reverse transcriptase gene from plasma HIV-1 RNA amplification products was sequenced. Resistance mutations in reverse transcriptase were present or absent as described in table [103, 115, 116, 254].

Statistics.

All statistical analyses (alpha level: 0.05) were performed using Prism v. 5 (Graph Pad Software). Kaplan-Meier plots indicate the percentage of animals that are HIV-1 positive in the peripheral blood at each time point analyzed. Power analysis calculation for experimental group sample sizes were determined as previously described [255, 256]. Briefly, we assumed 50 and 65% variance in transmission between our experimental groups for HIV-1_{JRCSF} and HIV-$1_{JRCSF(K65R)}$, respectively. In the case of each viral isolate, the chosen sample sizes were determined to have 90% power to detect statistically significant differences via log rank test analysis in the treatment arm versus the vehicle arm.

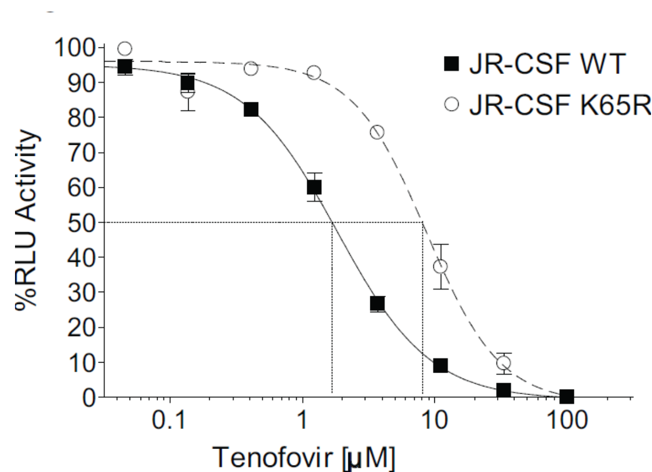

Figure 6. Introduction of K65R mutation into $HIV_{JR\text{-}CSF}$ results in a 4.7 fold increase of *in vitro* IC_{50} using a luciferase based assay in TZM-bl indicator cells.

Serial dilutions of tenofovir were applied to indicator cells in triplicate and allowed to incubate for 30 mins before an equal number of tissue culture infectious units (TCIU) of either wild type or mutant virus was applied to all wells. Two days later, the media is removed, ONE-GLO reagent (Promega) was added and the amount of luciferase activity was measured. Each curve was normalized to wells infected with that specific virus (wild type or K65R virus) in the absence of drug. RLU= relative light units.

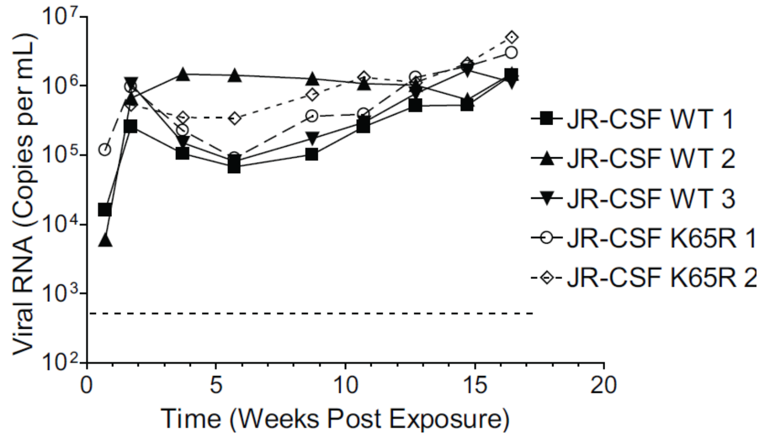

Figure 7. ***In vivo*** **replication of $HIV_{JR\text{-}CSF}$ and $HIV_{JR\text{-}CSF\ K65R}$ after IP injection into humanized mice shows no overt difference in replication capacity**

Humanized NOD/SCID/gamma$^{-/-}$ mice [261, 262] were infected with equal amounts of either HIV-$1_{JR\text{-}CSF}$ or HIV-$1_{JR\text{-}CSF\ K65R}$ ($3x10^4$ TCIU) by IP injection. The course of infection was monitored by determining plasma viral loads.

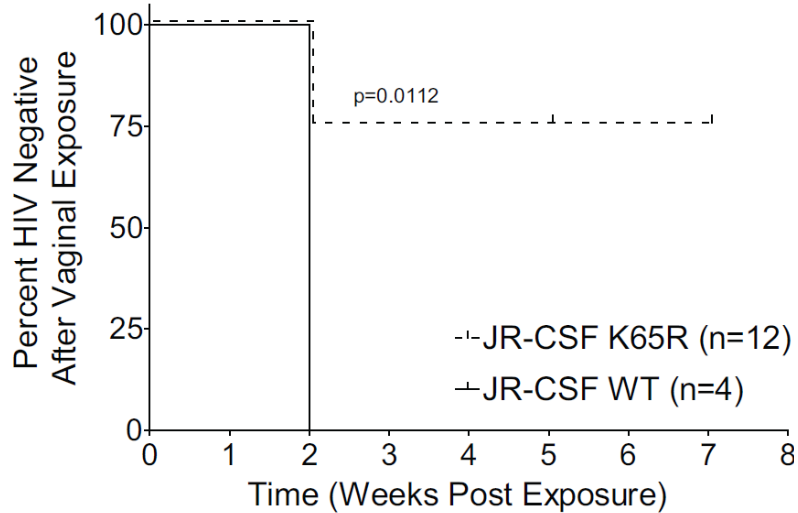

Figure 8. The K65R mutation reduces vaginal transmission efficiency of HIV-1 by 75%

Humanized BLT mice were prepared and validated as previously described [180, 185, 204]. Mice were exposed vaginally to a single dose of HIV-$1_{JR\text{-}CSF}$ or HIV-$1_{JR\text{-}CSF\ K65R}$ ($3.5x10^5$ TCIU). Infection was monitored as a function of viral load in plasma. Kaplan-Meyer plot represents the percentage of HIV negative mice as a function of the number of weeks post-exposure until the first peripheral blood HIV-1 detection. Of the mice exposed to wild type HIV_{JRCSF}, 4 of 4 mice had detectable viral RNA by week 2 post exposure. Of the mice exposed to the K65R mutant HIV, 3 of 12 (25%) mice viral load was readily detectable 2 weeks post exposure. The remaining 9 of12 mice (75%) mice exposed to the K65R mutant no viral load was detected at any time point analyzed and no viral DNA was found in tissues at harvest confirming lack of transmission.

TABLE 3. Sequence analysis demonstrates reversion of the K65R mutation over time in peripheral blood, cervicovaginal lavage and tissues of infected BLT mice*.

Vaginally infected by $HIV_{JR\text{-}CSFK65R}$	Week post exposure	Analysis of Viral Sequence from Bulk PCR Products at Position 65					
		Peripheral blood	Vaginal lavage	FRT	Lymph node	Organoid implant	Lung
M1	4	R only	n/a	n/a	K and R	K and R	K and R
M2	4	K only	n/a	n/a	K only	K only	K only
M3	4	K and R	K and R				
	6	K and R	K only				
	9	K only	K only				
	13	K only	K only				
	14	K only	K only	K only	K only	K and R	K only

*Bone marrow/liver/thymus mice were exposed once intravaginally once to mutant virus. Infection was monitored in plasma by determining viral load. Two mice were harvested four weeks post infection (M1 and M2) and one was harvested 14 weeks post exposure (M3). Peripheral blood and vaginal lavage samples from this mouse were collected longitudinally. Only FRT, female reproductive tract. K, lysine. R, arginine. PCR primer sets used to amplify RT: outer/first reaction: 5'-GCTCTATTAGATACAGGAGC-3', 5'-CCTAATGCATATTGTGAGTCTG-3', inner/second reaction :5'-GTAGGACCTACACCTGTCAAC-3', 5'-CCTGCAAAGCTAGGTGAATTGC-3'.Amplification products were sequenced in bulk.

CHAPTER IV. EVALUATION OF AN ENTRY INHIBITOR FOR USE AS PREP[3]

AUTHOR SUMMARY

Antiretroviral drugs from the entry inhibitor class have the potential to be highly effective at preventing transmission of HIV when used as a pre-exposure prophylaxis. Unlike tenofovir, entry inhibitors are not part of first line therapies which would reduce the likelihood of PrEP resulting in resistance to ART. Alternatively, combining drugs from multiple classes could increase the efficacy of PrEP and offer a wider spectrum of protection. For these reasons, we chose to evaluate the entry inhibitor Maraviroc for its potential to prevent HIV transmission. Utilizing BLT humanized mice, Maraviroc administered once a day orally prevented vaginal HIV transmission. One percent Maraviroc applied vaginally completely prevented vaginal HIV transmission. The same solution applied rectally also resulted in complete protection from rectal HIV transmission. In summary, these studies support the use of the entry inhibitor Maraviroc as an effective candidate drug for both oral and topical PrEP.

INTRODUCTION

As described in the previous chapters, the use of multiple antiretroviral drugs for PrEP strategies will help prevent the *in vivo* selection and the transmission of drug resistant HIV. In addition, utilization of multiple antiretroviral drugs has the potential to combine drugs with

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[3] The following work is unpublished data.

Author contributions are as follows: conceived and designed the experiments, MC, MS performed the experiments, MC ; analyzed the data, MC; predicted authors for the paper, MC, IM, VG

complementary PK/PD profiles, formulation possibilities, and could have an increased protective effect by targeting multiple steps of the viral replication cycle. Before developing a multiple drug combination for topical PrEP, it is important to establish that the individual drugs each contribute to protection. In addition to topical tenofovir, maraviroc has been recently suggested as a candidate for topical PrEP [267].

Maraviroc is an antiretroviral drug that prevents HIV from binding to the CCR5 receptor. Nearly all new infections are the result of R5-tropic HIV transmission [243, 244]. When used for treatment, Maraviroc has the potential for inducing outgrowth of X4-tropic HIV, a viral tropism associated with a more pathogenic infection [268-271]. While studies have shown that treatment with Maraviroc does lead to outgrowth of X4-tropic or dual-tropic HIV, there are conflicting reports over whether these infections result in increased CD4 decline [268, 269, 272, 273]. In addition, patients who discontinue Maraviroc often see a reappearance of R5-tropic virus as the dominant viral population. While Maraviroc has been approved for ART, it is not a first line therapy drug. In theory, a person using Maraviroc as PrEP could experiences a breakthrough infection and the virus may develop resistance to Maraviroc but will maintain sensitivity to first line therapy drugs.

Pre-clinical trials have found Maraviroc to be protective in humanized mouse models. In a 2010 article, RAGhu mice were given Maraviroc once a day by oral gavage for seven days. On the fourth day, mice were vaginally exposed to HIV_{BAL-1}. Mice receiving Maraviroc had100% protection compared to the control mice. In a 2011 article, RAG-hu mice received a single treatment of a 5mM Maraviroc gel applied vaginally and an hour later were vaginally exposed to HIV_{BAL-1}. Mice that received the Maraviroc gel were completely protected from vaginal HIV

transmission while those that received the placebo gel all became infected. These studies support the use of Maraviroc to prevent vaginal HIV transmission whether it is given orally or topically.

In macaque models, a 3.3% MVC (by wt) gel was able tocompletey prevent vaginal transmsssion of SIV [274]. In this study, a single application of 3.3% MVC gel resulted in peak vaginal fluid drug levels at .5-2hrs after application (10^4 to 10^7 ng/mL) and drug was detectable for up to 72 hours [274]. On the other hand, a different study found no protection from rectal exposure when maraviroc was administered orally even though high levels of drug were detected in rectal tissue (1.4 ug MVC/g tissue) [275].

Building on these studies, we decided to evaluate Maraviroc as a oral or topical PrEP to prevent vaginal or rectal transmission of HIV in the BLT humanized mouse model. We utilized the CCR5-tropic, transmitted founder virus HIV_{THRO}. Transmitted founder viruses represent the most biologically relevant, molecular clones of HIV available for PrEP studies.

My results demonstrated protection against vaginal transmission when MVC was given as a daily oral dose. One percent Maraviroc applied topically resulted in complete protection of HIV transmission when applied at the site of exposure. These studies support the use of this entry inhibitor for dual compartment use in either oral or topical formulations.

RESULTS

Daily oral Maraviroc prevents vaginal transmission of HIV

To test the ability of Maraviroc taken orally to prevent HIV transmission, BLT mice (n=4) were administeed Maraviroc (62 mg/kg body weight) via oral gavage once a day for seven days. On the third day, the mice were vaginally exposed to $3.5x10^5$ TCIU HIV_{THRO}. Only 25% (1 out of 4) of the mice receiving Maraviroc became infected with HIV resulting in a significant

level of protection. All of the mice in the control arm became infected with HIV (n=5). (Figure 10).

Single pre-exposure application of 1% Maraviroc prevents vaginal transmission of HIV

To test the ability of Maraviroc applied topically to prevent vaginal HIV_{THRO} transmission, BLT mice (n=4) were administered 1% MVC (≈19mM) vaginally once within 30 minutes prior to HIV exposure. There was complete protection with statistical significance (Figure 11). This provides supporting evidence for development of an efficient Maraviroc based topical microbicide to prevent vaginal HIV transmission.

Single pre-exposure application of 1% Maraviroc prevents rectal transmission of HIV

To test the ability of Maraviroc applied topically to prevent rectal HIV_{THRO} transmission, BLT mice were administered a single dose of 1% MVC topically within 30 minutes prior to HIV exposure. Mice were given MVC rectally and were exposed rectally. There was complete protection with statistical significance (Figure 13). This provides supporting evidence for development of an efficient Maraviroc based topical microbicide for rectal application.

DISCUSSION

There is great support and building evidence that antiretroviral drugs can effectively prevent HIV transmission [18, 23, 180, 184, 265, 276, 277]. To date, the greatest progress has been made with Tenofovir containing strategies. The dual use of Tenofovir for prevention and as first line therapy has raised concerns including *de novo* and transmitted tenofovir resistance.

These concerns can be addressed by utilization of multidrug PrEP and/or use of an antiretroviral drug not commonly used for first line therapy. Antiretroviral drugs from the entry inhibitor class are not usually prescribed for first or second line therapy thereby making them excellent candidates for PrEP studies.

For my studies, the entry inhibitor Maraviroc was evaluated as for PrEP when administered orally or topically. Maraviroc is a negative allosteric modulator of the CCR5 receptor which results in an inability to bind the HIV gp120 protein [278]. Despite being very potent and having a good safety profile, Maraviroc is not a first line therapy drug. In terms of HIV transmission, the virus transmitted through sexual encounters is almost exclusively CCR5 tropic with some evidence of occasional dual tropic virus transmission. Therefore, Maraviroc is an excellent candidate drug for HIV PrEP prevention strategies.

Previously published work in the RAG-hu humanized mouse model has shown Maraviroc to be protective against vaginal HIV transmission when administered orally or topically [277, 279]. In addition, a recently published study using topical Maraviroc in a 3% by wt gel found complete protection in macaque model when used vaginally [274]. Following these studies, it was reported that Maraviroc administered orally had no protective effect in a non-human primate model of rectal transmission despite detectable levels of drug in rectal tissue [275]. My results agree with the previous humanized mouse studies concerning vaginal transmission and, in addition, demonstrates protection from rectal transmission if Maraviroc is used topically.

Maraviroc is one of the few anti-HIV drugs that targets a host protein instead of targeting to a viral protein.. The human and rhesus CCR5 differs by eight amino acid residues. In 2012, Malcolm et. al. measured the EC_{50} values for Maraviroc when used with human or rhesus

PBMCs. The authors reported a 5 fold difference in EC_{50} when using SHIV-162P3 and a 10 fold difference in EC_{50} when using SIV-mac251 [280]. These head-to-*head in vitro* comparisons indicate that Maraviroc is less effective at protecting rhesus cells from infection and more effective at protecting human cells from infection. Therefore, Maraviroc may not be as effective in experimental models where the CCR5 receptor is not human

In conclusion, my research supports the use of Maraviroc for effective HIV prevention as either an orally administered drug or as a topical application. Orally administered Maraviroc provided 75% protection against vaginal HIV transmission. Topically administered Maraviroc was able to provide complete protection from either vaginal or rectal exposure making it a good candidate for a dual use microbicide.

MATERIAL AND METHODS

Preparation of BLT mice and characterization of human reconstitution.

BLT mice were prepared essentially as previously described [178-185, 187, 200]. Briefly, thy/liv implanted [250] and preconditioned NOD/SCID-gamma chain null (NSG) mice (Jackson Laboratories, Bar Harbor, ME) were transplanted with autologous human fetal liver $CD34^+$ cells (Advanced Bioscience Resources, Alameda, CA) and monitored for human reconstitution in peripheral blood by flow cytometry [183, 185, 187]. All BLT mice used herein (n=31) had high peripheral blood reconstitution levels of human lymphoid ($CD45^+$) cells (74% mean ± 10%, SD) and human $CD4^+$ T cells (82% mean ± 4%, SD) (Summarized in Figures 9 and 12).Mice were maintained at the University of North Carolina at Chapel Hill Division of Laboratory Animal

Medicine in accordance with protocols approved by the Institutional Animal Care and Use Committee.

MVC solution preparation

Maraviroc was purchased from Selleckchem (Cat. S2003, MW 513.67g/mol, >99% purity). Maraviroc powder was added to PBS and 1M HCL was added until MVC fully went into solution. The pH was then adjusted to neutral using dilute NaOH. Finally, PBS was added to solution until reaching a 1% by weight concentration of Maraviroc. The final solution was sterile filtered using Nalgen .2μ luer lock filters. The same solution was utilized for both oral gavage and topical administration.

Oral dosing of Maraviroc and vaginal exposure of BLT mice to HIV-1

BLT mice in the Maraviroc drug arm received a daily oral gavage of 1.23mg MVC per 20 gram mouse weight daily for seven days resulting in a total of seven gavages. Two hours after the third gavage, the mice were vaginally exposed to $3.5x10^5$ TCIU of HIV_{THRO}.

Topical application of tenofovir and vaginal or rectal exposure of BLT mice to HIV-1.

A stock of HIV-1_{THRO} [243] was prepared and titered as we have previously described [181, 252]. Mice were exposed rectally using 0.7 μg p24 of HIV-1_{THRO} ($5x10^6$ TCIU). Topical Maraviroc consisted of 1% Maraviroc by weight in PBS. The vehicle (placebo) control was PBS.

The exposure timeline consisted of rectal application of vehicle or of 1% Maraviroc less than 30 minutes prior to rectal application of virus. Rectal exposures with HIV-1_{THRO} were performed essentially as previously described [184, 187] except that all the mucosal exposures were carried out atraumatically and without simulated rectal intercourse [253]. All rectal applications of vehicle or inhibitor as well as virus were performed while mice were anesthetized

[184, 187]. After viral exposure, mice were returned to their housing to recover and were then monitored longitudinally for evidence of HIV-1 infection as indicated below.

Analysis of HIV-1 infection of BLT mice.

Infection of BLT mice with HIV-1 was monitored at the indicated time intervals in peripheral blood by determining plasma levels of viral RNA using real time PCR (limit of detection 750 copies/ml) [179, 180] and by monitoring $CD4^+$ T cell percentages by flow cytometry [183, 184]. At necropsy, tissues were harvested and mononuclear cells isolated as previously described [178, 180, 183, 185, 187]. Mononuclear cells were washed, enumerated and tested using real time PCR for the presence of HIV-1 DNA (limit of detection 10 copies) [180, 181, 183, 184].

Statistics.

All statistical analyses (alpha level: 0.05) were performed using Prism v. 5 (Graph Pad Software). Kaplan-Meier plots indicate the percentage of animals that are HIV-1 negative in the peripheral blood at each time point analyzed.

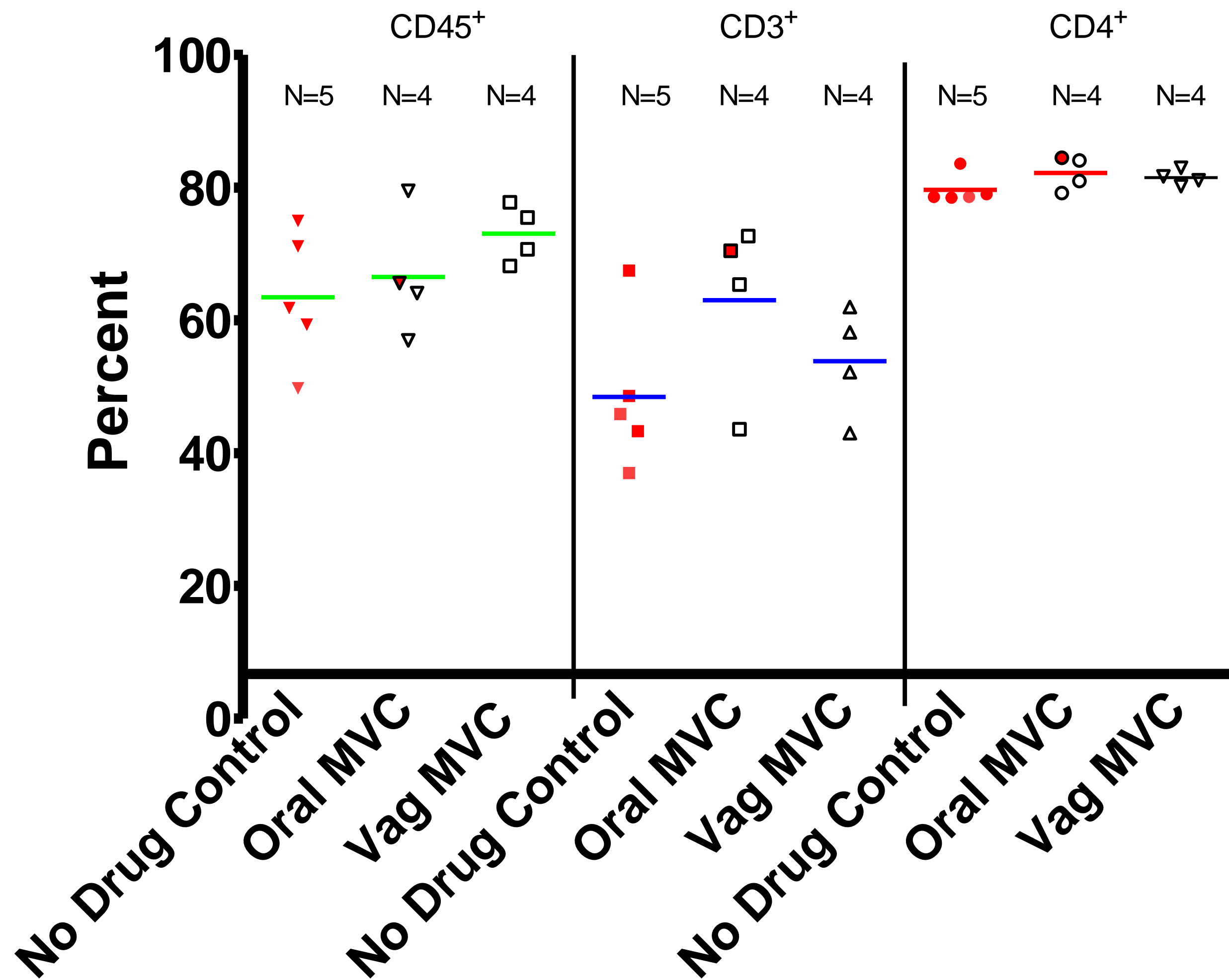

Figure 9. Characterization of the levels of human cell types present in peripheral blood of BLT mice used in vaginal exposure studies

Prior to vaginal exposure, the peripheral blood of each mouse was analyzed by flow cytometry for the presence of human hematopoietic cells (CD45, green bar represents mean), human T-cells (CD3, blue bar represents mean), and human CD4+ T cells (CD4, red bar represents mean). Mice are grouped by experimental arm: no drug controls (PBS), daily oral MVC administration (Oral MVC), and single application of topical 1% MVC (Vag MVC). Data points depicted in red represent animals that became infected with HIV. Overall, the mice in all experimental arms had comparable in levels of human cells (t-test).

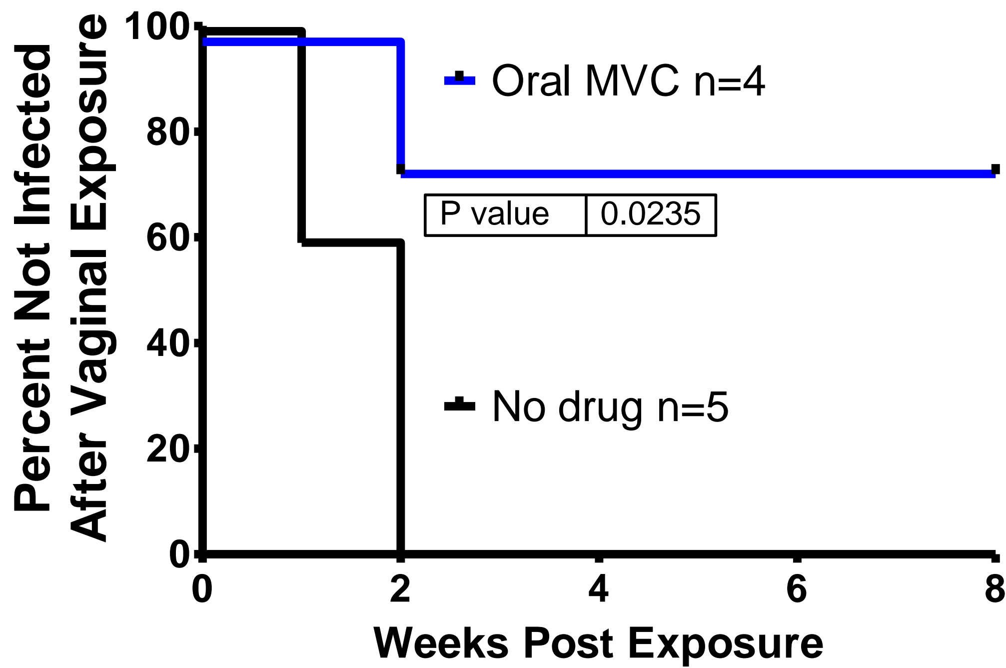

Figure 10. Orally administered Maraviroc prevents vaginal transmission of HIV

Kaplan-Meier plot indicates the time to detection of viral RNA or DNA following vaginal HIV-1_{THRO} exposure in BLT mice pretreated with either vehicle or oral Maraviroc. Log-rank (Mantel Cox) analysis reveals a statistically significant difference in vaginal HIV-1_{THRO} transmission between the vehicle and oral Maraviroc arms.

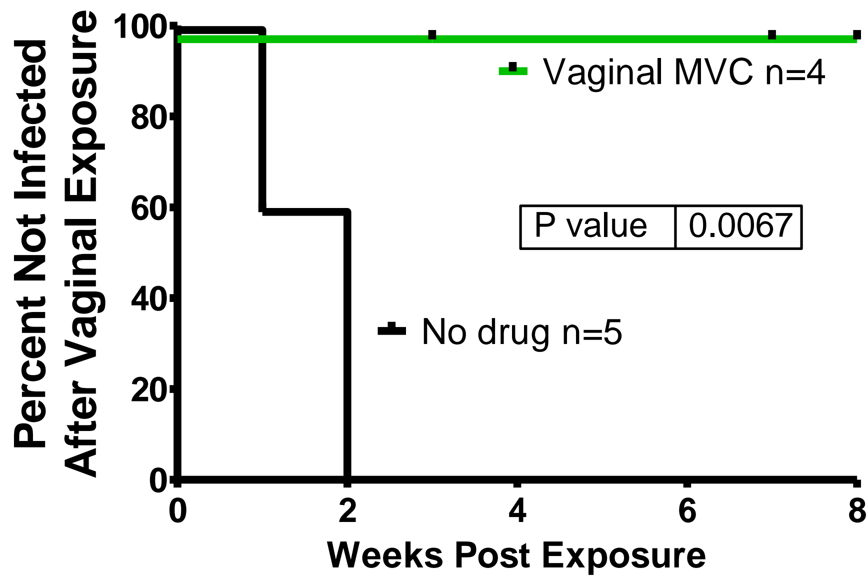

Figure 11. A single application of 1% Maraviroc prevents vaginal transmission of HIV

Kaplan-Meier plot indicates the time to detection of viral RNA or DNA following vaginal HIV-1_{THRO} exposure in BLT mice pretreated with either vehicle or topical MVC. Log-rank (Mantel Cox) analysis reveals a statistically significant difference in vaginal HIV-1_{THRO} transmission between the vehicle and topical MVC arms.

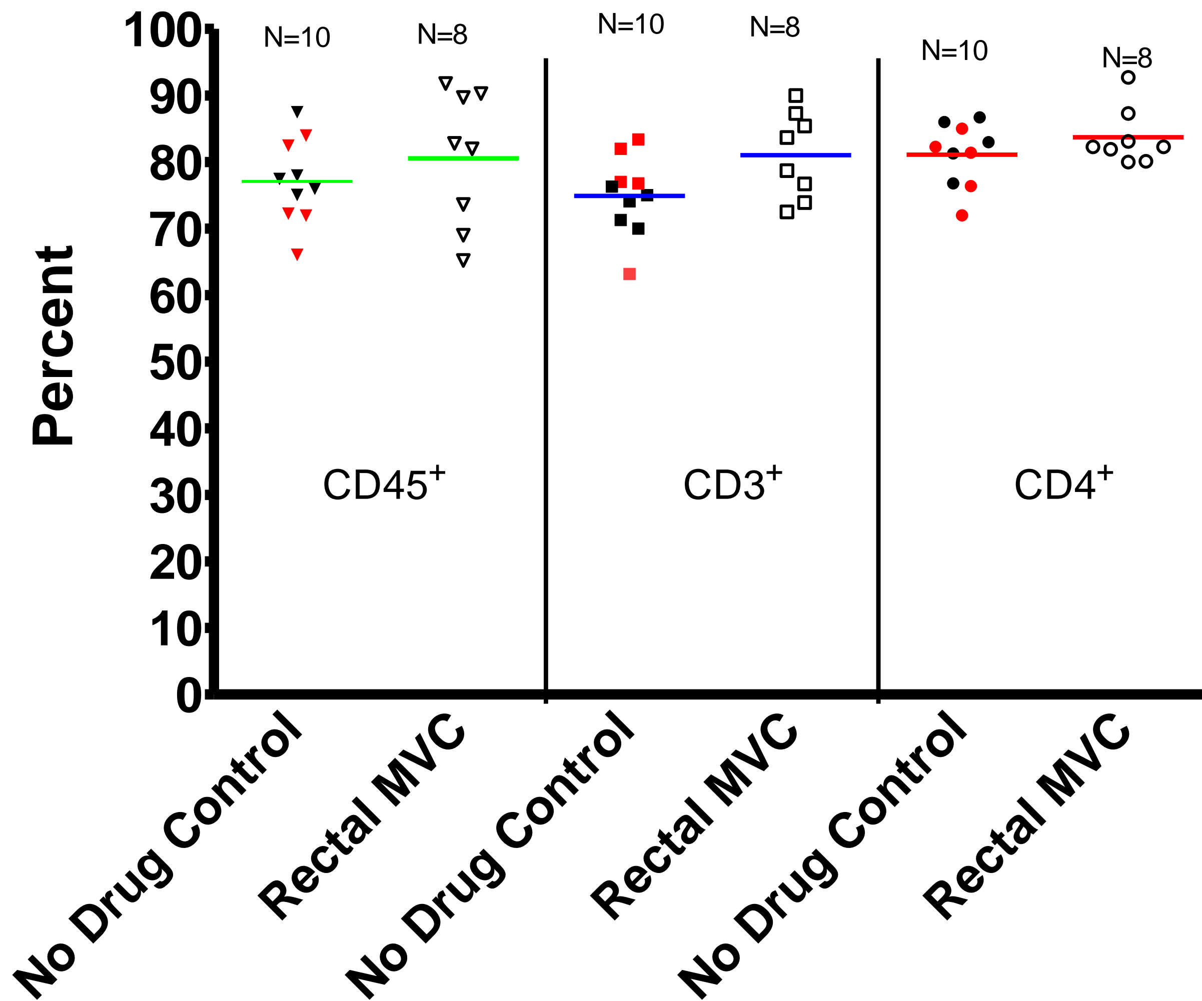

Figure 12. Characterization of the levels of human cell types present in peripheral blood of BLT mice used in rectal exposure studies

Prior to rectal exposure, the peripheral blood of each mouse was analyzed by flow cytometry for the presence of human hematopoietic cells (CD45, green bar represents mean), human T-cells (CD3, blue bar represents mean), and human CD4+ T cells (CD4, red bar represents mean). Mice are grouped by experimental arm: no drug controls (PBS) or a single application of topical 1% MVC (MVC). Data points depicted in red represent animals that became infected with HIV. Overall, the mice in all experimental arms were comparable in humanization.

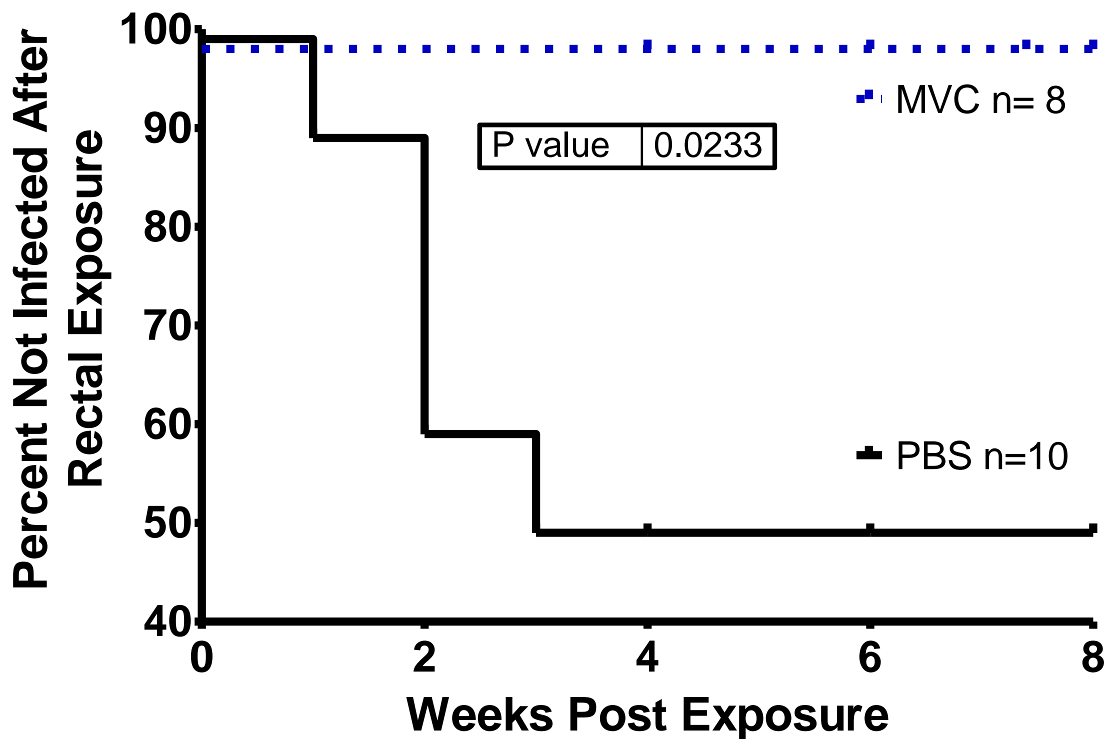

Figure 13. A single topical application of 1% Maraviroc prevents rectal transmission of HIV

Kaplan-Meier plot indicates the time to detection of viral RNA or DNA conversion following rectal HIV-1_{THRO} exposure in BLT mice pretreated with either vehicle or topical Maraviroc. Log-rank (Mantel Cox) analysis reveals a statistically significant difference in rectal HIV-1_{THRO} transmission between the vehicle and topical Maraviroc arms.

CHAPTER V. EFFECT OF MICROBIOTA ON HIV TRANSMISSION[4]

AUTHOR SUMMARY

The microbiome/microbiota is the aggregate of microorganisms that resides on the surface and within the host's tissues. Each host species contains a microbiome that is unique to the host and has co-evolved with that particular species [281, 282]. Fluctuations in microbiome composition can exacerbate or suppress immune responses [282, 283]. Microbiome composition can affect transmissibility and pathogenesis of infectious agents and opportunistic pathogens [151, 284, 285]. Observational studies have suggested that microbiome composition may affect HIV transmission and pathogenesis [145, 154, 157]. For this reason, the following work describes the first small animal model available to evaluate HIV transmission and pathogenesis in the context of differing microbiota. Humanized mice provide the unique ability to evaluate the *in vivo* function of the human immune system in total absence of microbiota or with a few select organisms. To achieve this goal, NSG mice were derived in germ free conditions and used to establish a germ free NSG colony. These mice were used to generate humanized with a human associated microbiome environment. Preliminary results showed that humanized mice with a human microbiome have an increased HIV transmission efficiency than humanized mice with a murine microbiome. These mice will provide a powerful tool for evaluating the human immune system's response to the presence or absence of a microbiome. We will also evaluate the microbiome effect on viral load and $CD4^+$ cell decline.

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[4]The following is unpublished data

Author contributions are as follows: conceived and designed the experiments, MC VG; performed the experiments, MC ; analyzed the data, MC; predicted authors for the paper, MC, IM, VG

INTRODUCTION

Microbiome may affect HIV transmission and pathogenesis

Several clinical studies have linked microbial populations with HIV transmission and pathogenesis [149, 157, 158, 286-288]. Co infections with STDs such as that cause disruption of the epithelia layer, genital herpes, or that cause local inflammation, yeast infection, tend to increase the likelihood of acquiring an HIV infection [289, 290]. On the other hand, it is predicted that the presence of Lactobacillus species will reduce the transmission of HIV [161, 291]. Therefore, the treatment of current STDs or the use of probiotics may be a way to actively reduce HIV transmission.

Microbial populations have also been suspected for affecting HIV pathogenesis. $CD4^+$ T-cell decline is one of the defining characteristics of HIV infection. Chronic T-cell activation and death has been linked to microbial translocation in the intestinal tract [157, 287, 292-294]. Studies to evaluate the effect of microbial composition on HIV pathogenesis may provide unique insights into why some individuals progress to AIDS faster or slower than others [287]. Ultimately, HIV infection doesn't kill a person. The opportunistic infections associated with AIDS are the direct reason for death of untreated HIV infected patients [155].

Small animal model to study effect of microbiota on HIV transmission

As previously described, humanized mice are made from genetically immune deficient mice strains such as NSG. While some microbiome studies are done using massive antibiotic treatments to alter commensal microbiota composition, we wanted to create the perfect "empty

vessel" for our microbiome studies only achievable by using germ free animals [295, 296]. A germ free animal is specifically an animal completely devoid of all bacteria, all fungi, and the vast majority of viral species [297, 298]. These animals are born under sterile conditions and must remain housed in sterile isolators to maintain their germ free status [299]. We derived a germ-free NSG mouse colony *de novo* at the National Gnotobiotic Rodent Resource Center at University of North Carolina, Chapel Hill. Some germ-free NSG mice were removed from germ-free housing at the age of weaning and given a human microbiome by oral gavage with human feces. These human microbiota mice were the used to generate BLT humanized mice. Preliminary results show a higher rate of rectal HIV transmission in humanized mice that received human microbiota.

RESULTS

Different rates of HIV transmission occur at different animal housing locations

Prior to 2010, our mice were housed in the animal facilities of the University of Texas Southwestern Medical Center at Dallas, TX. At this facility we saw a constant rectal HIV transmission efficiency of 63-69% [184, 203]. None of the previous mice or animal equipment was relocated to the University of North Carolina Chapel Hill. Initially, at UNC, mice were purchased from Jackson Laboratories and were housed in the upper basement level of the Genetic Medicine Building (GMB) in a room that had previously housed animals but was emptied for our exclusive use (Room 35, upper basement). At this location, we had inconsistent rectal HIV transmission with the average transmission of 75% (62% to 100%) for mice rectally exposed to $5x10^6$ TCIU HIV_{THRO} (n=12). One year later, a brand new, never used before animal

housing space became available for our use in the lower basement of GMB (Room 35, lower basement). No animals were relocated to the lower basement. Rather new animals were purchased from Jackson Laboratories. At this new location, we had a severely reduced rate of rectal HIV transmission of 18% (0 to 50%) for mice rectally exposed to $5x10^6$ TCIU HIV_{THRO} (n=22). In response to this severe decrease in rectal transmissions, we wished to return to the upper basement housing (Room 35, upper basement) for our experiments but this room had been repurposed and was unavailable. As an alternative, we made use of animal housing in another room of the upper basement where multiple laboratories housed several strains of mice (Room 40, upper basement). Room 41 of the upper basement is a shared space with common use ventilated hoods for animal manipulations. For rectal exposures, BLT animals were generated in the lower basement Room 35 and relocated to the upper basement Room 40 where they were put on old bedding and allowed to acclimate to the housing conditions and local microbial populations for at least two weeks before exposure. Rectal HIV transmission was consistently 50% (50% every experiment) for mice rectally exposed to $5x10^6$ TCIU HIV_{THRO} in Room 40 of the GMB upper basement (n=10). All of these results are depicted in Figure 14. Taken together, we hypothesized that the greatest factor affecting rectal HIV transmission efficiency was the local microbiota present in each housing location.

Humanized mice with human microbiota have optimal rectal HIV transmission

All mice in the previous experiments were either supplied directly from Jackson Laboratories or bred in our housing from Jackson Laboratory mice. Therefore, we would expect the microbiota of our mice to be a mixture of the original microbiota acquired at Jackson breeding facility and an addition of some microbiota acquired in our housing.

To evaluate the effect of a human associated microbiome (HuMb) on HIV transmission, we inoculated germ free NSG mice via human fecal transplant and had no adverse events. These mice were housed in their own room separate from any other animal housing area. These mice were then humanized using the BLT protocol and exposed rectally to $5x10^6$ TCIU HIV_{THRO}. HuMb BLT mice had the highest transmission efficiency of all other housing conditions at 83% (n=6). These results suggest that HIV transmission in BLT mice is affected by the microbiome and that the human microbiome is optimal for HIV transmission in humanized mice. Further studies are needed to determine which components of the microbiome are responsible for the effect on HIV transmission.

DISCUSSION

HIV transmission primarily occurs at mucosal sites of sexual exposure. These sites are colonized with a community of commensal organisms some of which are opportunistic pathogens [300, 301]. Clinical trials evaluating HIV transmission have suggested that the composition of microbiota at these mucosal surfaces affects HIV transmission [145, 155, 157]. Unfortunately, HIV transmission studies involving humans can be inherently difficult. To address this issue, we developed a novel humanized mouse model capable of evaluating the effect of microbiota on HIV transmission and pathogenesis. Germ free NSG mice were colonized with a human microbiota (HuMb) and then given a human immune system (BLT) to create a HuMb BLT experimental model. HuMb BLT mice rectally exposed to HIV resulted in transmission of 83% of the mice. This transmission efficiency is higher than any previous BLT

mouse regardless of housing conditions. Therefore, we provide evidence showing the dramatic effect of microbial populations to affect HIV transmission.

HuMB BLT mice were also tested for vaginal and oral transmission of HIV-1. All mice exposed to HIV-$1_{JR\text{-}CSF}$ vaginally became infected (Figure 15). Mice orally exposed to HIV-1_{JRCSF} were also susceptible to infection (75% transmission, Figure 15). Further studies will be needed to determine the relative transmission efficiencies between HuMB BLT and BLT mice with a murine microbiome.

Future studies to be done with HuMb BLT mice include 1) determining if specific species or genus of organisms can be sufficient to increase HIV transmission in conventionally housed BLT mice, 2) determine if alterations in microbiome composition affects HIV pathogenesis, 3) determine if cohousing HuMb BLT mice with conventional MMb BLT mice affects transmission efficiency, and 4) determine how antibiotic or antifungal treatments affect microbiome composition and HIV transmission. In addition, the availability of germ free NSG mice gives us the opportunity to perform mono-colonization studies, probiotic studies, or test the efficacy of anti-HIV living microbicides under development.

MATERIALS AND METHODS

Derivation of germ free NSG mice

Germ free NSG mice were derived and housed at the National Gnotobiotic Rodent Resource Center at the University of North Carolina, Chapel Hill NC. Conventially housed NSG females received hormone injections to induce superovulation. These superovulated females were then co-housed with male NSG mice and fertilization was verified by the presence of a

sperm plug. NSG embryos were removed under sterile conditions and evaluated *ex vivo* for quality. High quality embryos were transferred under sterile conditions into a germ free, surrogate female mouse. The surrogate mother carried the NSG embryos to term and gave birth to a litter of germ free NSG pups. Brother sister mating was used to found a colony of germ free NSG mice.

Colonization of germ free mice with human microbiota

A human fecal sample was obtained from a healthy adult donor and homogenized with PBS before freezing 97 aliquots in liquid nitrogen storage. An aliquot was sent to Charles River for PCR analysis to ensure the absence of any mouse or human pathogens. Germ free mice were removed from sterile housing and immediately were administered a human fecal transplant. Fecal transplants were performed by thawing one aliquot and first gavaging the animals followed by topical application to all four paws and the urogenital region.

Preparation of BLT and NSGhu mice and characterization of human reconstitution.

BLT mice were prepared essentially as previously described [178-185, 187, 200]. Briefly, thy/liv implanted [250] and preconditioned NOD/SCID-gamma chain null (NSG) mice (Jackson Laboratories, Bar Harbor, ME) were transplanted with autologous human fetal liver $CD34^+$ cells (Advanced Bioscience Resources, Alameda, CA) and monitored for human reconstitution in peripheral blood by flow cytometry [183, 185, 187]. Mice were maintained at the University of

North Carolina at Chapel Hill Division of Laboratory Animal Medicine in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Rectal exposure of HuMb BLT mice to HIV-1.

A stock of HIV-1_{THRO} [243] was prepared and titered as we have previously described [181, 252]. Mice were exposed rectally using 0.7 μg p24 of HIV-1_{THRO} ($5x10^6$ TCIU). Rectal exposures with HIV-1_{THRO} were performed essentially as previously described [184, 187] except that all the mucosal exposures were carried out atraumatically and without simulated rectal intercourse [253]. All rectal applications of vehicle or inhibitor as well as virus were performed while mice were anesthetized [184, 187]. After viral exposure, mice were returned to their housing to recover and were then monitored longitudinally for evidence of HIV-1 infection as indicated below (Figure 14).

Vaginal or Oral exposure of HuMb BLT mice to HIV-1.

A stock of HIV-1_{JR-CSF} [243] was prepared and titered as we have previously described [181, 252]. Mice were exposed vaginally using 0.04 μg p24 of HIV-1_{JR-CSF} ($3x10^5$ TCIU) atraumatically.Mice were exposed orally using 0.22 ug p24 HIV-11_{JR-CSF} ($1.6x10^6$ TCIU) . After viral exposure, mice were returned to their housing to recover and were then monitored longitudinally for evidence of HIV-1 infection as indicated below (Figure 15).

Analysis of HIV-1 infection of BLT mice.

Infection of BLT mice with HIV-1 was monitored at the indicated time intervals in peripheral blood by determining plasma levels of viral RNA using real time PCR (limit of detection 750 copies/ml) [179, 180] and by monitoring $CD4^+$ T cell percentages by flow cytometry [183, 184]. At necropsy, tissues were harvested and mononuclear cells isolated as previously described [178, 180, 183, 185, 187]. Mononuclear cells were washed, enumerated and tested using real time PCR for the presence of HIV-1 DNA (limit of detection 10 copies) [180, 181, 183, 184].

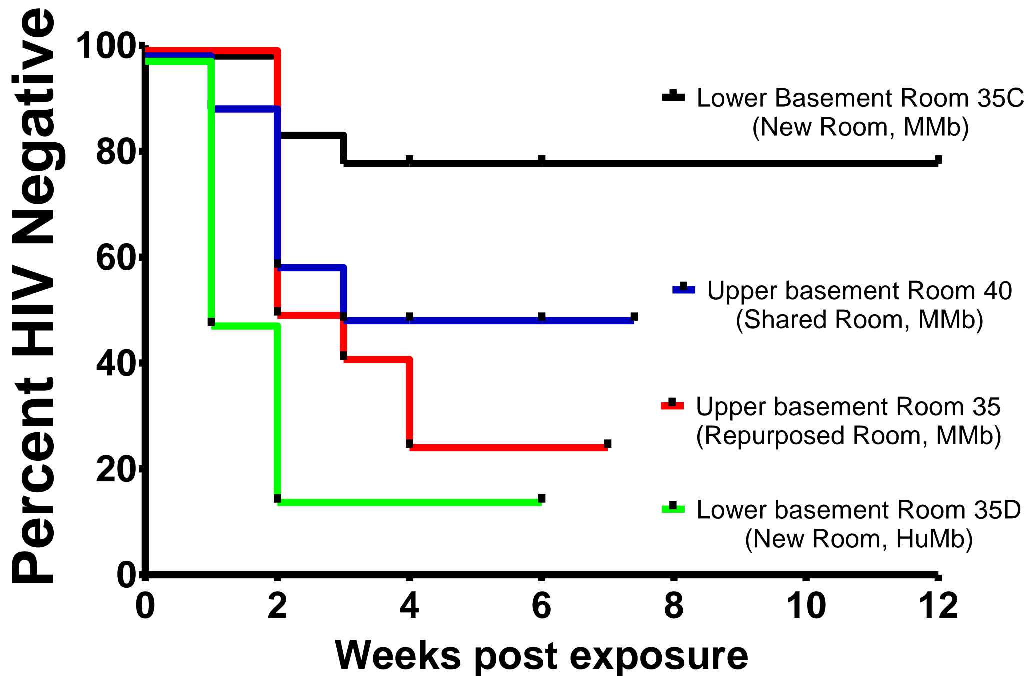

Figure 14 BLT mice with human microbiome have highest rectal HIV transmission efficiency

Mice were housed in four physically distinct animal rooms at the University of North Carolina, Genetic Medicine Building (GMB) animal facilities. GMB consists of two basement levels: the upper basement and the lower basement. Within these levels, the animal housing space is divided into a series of multi-room suites. Upper basement Room 40 (blue line, n=10) houses multiple strains of SPF mice from multiple laboratories and has communal air controlled hoods for animal housing. Upper basement Room 35 (red line, n =12) previously housed multiple strains of mice but was decontaminated and repourpased for our exclusive use. Lower basement Room 35C (black line, n=22) was a newly constructed space. Mice in the lower basement were received directly from Jackson Laboratories or bred in house in the lower basement. Lower basement Room 35D (green line, n=6) is a separate room used exclusively to house mice with a human microbiome.

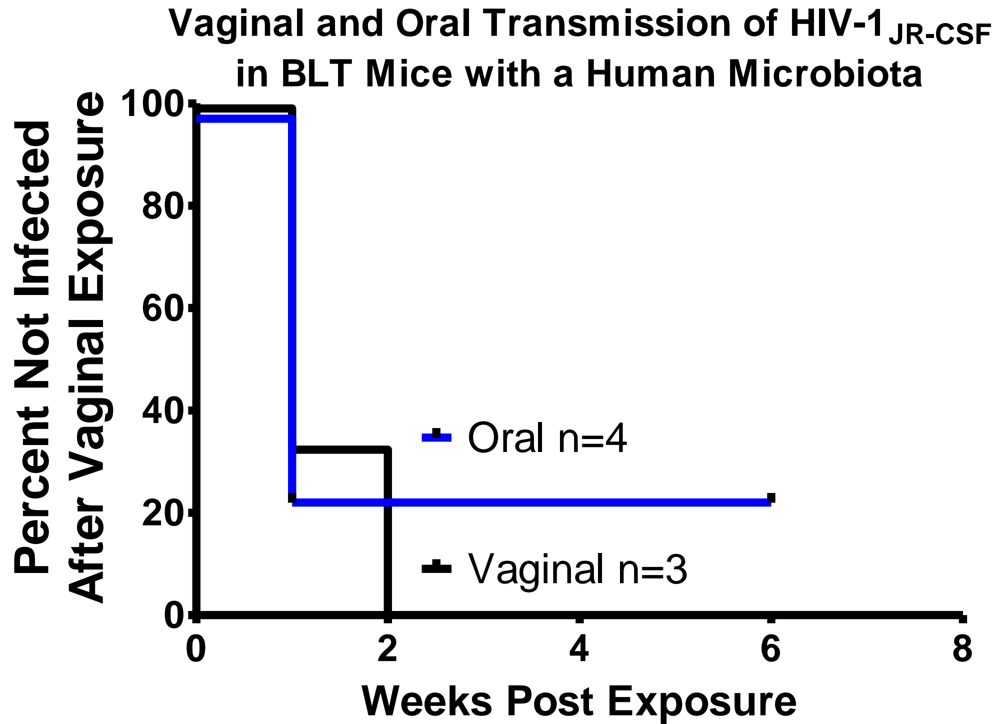

Figure 15 BLT mice with human microbiome efficiently transmit HIV via vaginal or oral routes

Human microbiome BLT mice were exposed vaginally (black line) or orally (blue line) to $3x10^5$ TCIU HIV-$1_{JR-CSF.}$ Human microbiome BLT mice were exposed orally (blue line) to $1.4x10^6$ TCIU HIV-1_{JR-CSF} Peripheral blood was evaluated for detectable viral RNA each week post exposure.

CHAPTER VI. DISCUSSION AND FURTHER DIRECTIONS

In absence of a reliable cure or vaccine, transmission prevention is the only method available to contain the HIV/AIDS epidemic. Sexual transmission is the main contributor to HIV spread globally. Previous methods to prevent sexual transmission of HIV, such as male condoms, require consent and acceptance of the penetrive partner. Recently, Truvada was approved for use as oral PrEP but may not be an option for patients on other medications, who are unlikely to adhere to a daily regimen, or who simply do not prefer to take oral pills. Therefore, alternative methods such as topical microbicides may be an acceptable and effective addition to current HIV prevention options.

My work has focused on evaluating novel methods to prevent HIV transmission at mucosal sites. *In vivo* experiments utilizing humanized mice demonstrated that both vaginal and rectal HIV transmission can be prevented by utilizing topically applied antiretroviral drugs such as tenofovir or Maraviroc. Transmission of tenofovir resistant HIV was found to be greatly reduced when compared to wildtype virus. The transmitted founder virus HIV_{THRO} was found to transmit and replicate as well as the previously used viral strain common for laboratory research HIV_{JRCSF}. Finally, a completely novel line of investigation was initiated by generating a humanized mouse model colonized with a human microbiome, HuMb BLT. This mouse model resulted in a higher rate of rectal transmission efficiency than any previous BLT model. Taken together, my work contributes to the immediate development of antiretroviral based PrEP strategies and can play a role in the novel investigations of microbial effect on HIV transmission and pathogenesis.

Truvada for PrEP is the most recent development for HIV prevention and it will take years before its efficacy outside of clinical trials can be evaluated. Some of the questions to be answered include 1) What is the minimum adherence to Truvada needed to achieve significant protection? 2) Should there be a breakthrough infection, will the person taking Truvada as PrEP be at significant risk of developing antiretroviral resistance? 3) Most of all, will the people who are at greatest risk of HIV actually use Truvada as PrEP? Most of these questions will be answered in time.

There is strong support to develop more ART based PrEP strategies. As discussed in previous chapters, one direction of research is to develop a topical microbicide to deliver antiretroviral drugs to the vaginal or rectal mucosa prior to HIV exposure. A major benefit of this approach is that it parallels the already prevalent use of sexual lubricants [302, 303]. Therefore, acceptability could be very high [303, 304]. In addition, topical microbicides could be used peri-coitally and may be effective immediately upon application as opposed to oral drugs which need time to reach the exposed tissues [31, 84, 274, 305, 306]. A drawback of topically administered PrEP is the greater potential for user error in dosing, proper application, etc. Therefore, it will be important to determine whether the efficacy of topical PrEP can overcome user error? Using a highly concentrated amount of drugs in topical formulations may ensure that even a partial dose will result in sufficient amount of drug presence to prevent HIV transmission. In fact, it has already been shown that topical administration of tenofovir results in far higher tissue concentrations than if the patient received tenofovir orally [84]. Topical administration of antiretroviral drugs may not result in the same side effects as orally administered drugs, such as bone mineral loss and interactions with other medications [307, 308]. Truvada for PrEP necessitates constant monitoring for breakthrough infection due to fear of breakthrough infection

followed by development of drug resistance. If a person has a breakthrough HIV infection while using topical PrEP, will they develop drug resistance? I believe this to be very unlikely due to the low to undetectable presence of ART drugs in the blood stream of patients using topical PrEP as well as the short duration of ART exposure compared to daily dosing of orally administered ART. This is a matter that can be addressed in animal models. If the concern for *de novo* drug resistance is eliminated, would it be possible for topical PrEP to be available over-the-counter? As mentioned before, acceptability is a large factor limiting current HIV prevention methods. Eliminating the need for doctor prescription would expand the availability of topical PrEP to at risk individuals who do not regularly see a doctor (i.e. healthy, young adults) and to individuals unlikely to ask a doctor for PrEP due to stigma. One could argue that over the counter dispensation removes a linkage to care. To which the counter argument is that these individuals had no linkage to care in the first place, nothing is being removed.

Long acting injectable drugs are under investigation for both HIV treatment and for protection [98]. Ideally, a patient receives an injection once a month and there is a continual, slow release of ART drugs over time sufficient to suppress ongoing infection or provide lasting protection against HIV acquisition. These could result in far better adherence than daily oral pills where accidently missing a dose is common. In terms of acceptability, industrialized nations tend to prefer pills to injections but this varies from individual to individual. In sub Saharan Africa, there is a strong precedent in place for long acting injectable birth control. Therefore, it may be a simple matter to pair the use of long acting injectable birth control with the use of long acting injectable HIV PrEP. This could be construed as a female-empowering form of HIV prevention given that the current most effective preventatives (condoms, male circumcision) require penetrive partner consent. A danger of long acting injectable ART is development of drug

resistance and side effects. Up to this point, any PrEP or ART can be effectively discontinued or altered upon the onset of drug resistance or side effects. Some long acting injectable drugs on the other hand may not be removed after injection. Should there be a breakthrough infection that is not fully suppressed by the injected ART, additional drugs will need to be added to prevent development of drug resistance. There is also the basic concern of adverse reactions to the drugs themselves. Ideally, a person could take an oral formulation of these drugs and monitor for adverse reactions prior to receiving the long acting inject able form.

The use of living microbes to prevent HIV transmission is the least developed of strategies due in part to its novelty and in part to the inherent difficulty of getting approvals to use self-replicating and/or genetically modified organisms in medicine. The idea of a living microbicide that can self replicate and potentially be spread to any sexual partner is both attractive and intimidating. The attraction of a living microbicide is that, in theory, administration to a single at risk individual will provide lasting protection to that individual and spread to all of their partners who will in turn transmit the organisms to their partners, much like an STD. Therefore, the living microbicide would reach the sexually active population most at risk of HIV infection without any need of administrators seeking them out. On the other hand, if an effective anti-HIV biological species is engineered to survive the intensely competitive vaginal and rectal compartments, is there a concern that this engineered species will then out grow and become invasive to the microbial community, resulting in a symptomatic dysbiosis. So far, this has not been the case in the studies evaluating these novel living microbicides. Actually, investigators have noticed that any administered biological organism is eventually out competed by indigenous species [309, 310]. While probiotics are a blossoming field with great potential, it

would take a great amount of time and data for a living HIV microbicide to take the place of current antiretroviral PrEPs under investigation.

In summary, the HIV epidemic is mainly perpetuated by sexual transmission despite currently available HIV prevention methods. Current popular HIV prevention methods require acceptance of the penetrating partner. While Truvada has been approved for PrEP, it is too early to determine if it will be effective outside of clinical settings. Treatment as PrEP is highly effective but requires a positive HIV status and a willingness to take ART early. In short, new preventative methods are needed that can be used by the penetrated partner unbeknownst to the penetrating partner or that are more acceptable that condom use. Topical microbicides are under investigation as HIV PrEP and are gaining support for approval. Improvements in formulations and more clinical trials are necessary, but previous trials have found a high level of acceptability for microbicides [311]. Ultimately, HIV prevention strategies can only succeed if they are used. Therefore, acceptability is key to stemming the HIV epidemic.

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