



Published in final edited form as:

Immunohorizons. ; 4(2): 72–81. doi:10.4049/immunohorizons.1900077.

Norethisterone Enanthate Increases Mouse Susceptibility to Genital Infection with Herpes Simplex Virus Type 2 and HIV Type 1

Nirk E. Quispe Calla^{*}, Rodolfo D. Vicetti Miguel^{*}, Angelo R. Torres[†], Wayne Trout[‡], Janelle M. Gabriel[§], Alissa M. Hatfield^{*}, Kristen M. Aceves^{*}, Jesse J. Kwiek[¶], Balveen Kaur^{||}, Thomas L. Cherpes^{*}

^{*}Department of Comparative Medicine, Stanford University, Stanford, CA 94305;

[†]Midwestern University College of Veterinary Medicine, Glendale, AZ 85308;

[‡]Department of Obstetrics and Gynecology, The Ohio State University, Columbus, OH 43210;

[§]School of Health and Rehabilitation Sciences, The Ohio State University, Columbus, OH 43210;

[¶]Department of Microbiology, The Ohio State University, Columbus, OH 43210;

^{||}Vivian L. Smith Department of Neurosurgery, McGovern Medical School, University of Texas, Houston, TX 77058

Abstract

Norethisterone enanthate (NET-EN) and depot-medroxyprogesterone acetate (DMPA) are two forms of injectable progestin used for contraception. Whereas clinical research indicates that women using DMPA are more susceptible to HIV and other genital pathogens, causal relationships have not been determined. Providing an underlying mechanism for this connection, however, is recent work that showed DMPA weakens genital mucosal barrier function in mice and humans and respectively promotes susceptibility of wild-type and humanized mice to genital infection with HSV type 2 and HIV type 1. However, analogous effects of NET-EN treatment on antiviral immunity and host susceptibility to genital infection are much less explored. In this study, we show that compared with mice in estrus, treatment of mice with DMPA or NET-EN significantly decreased genital levels of the cell–cell adhesion molecule desmoglein-1 and increased genital mucosal permeability. These effects, however, were more pronounced in DMPA- versus NET-EN–treated mice. Likewise, we detected comparable mortality rates in DMPA- and NET-EN–treated wild-type and humanized mice after intravaginal infection with HSV type 2 or cell-associated HIV type 1, respectively, but NET-EN treatment was associated with slower onset of HSV-induced genital pathology and lower burden of systemic HIV disease. These findings reveal DMPA and NET-EN treatment of mice significantly reduces genital desmoglein-1 levels and increases genital

This article is distributed under the terms of the [CC BY-NC-ND 4.0 Unported license](https://creativecommons.org/licenses/by-nc-nd/4.0/).

Address correspondence and reprint requests to: Dr. Nirk E. Quispe Calla and Dr. Rodolfo D. Vicetti Miguel, Stanford University School of Medicine, Edwards Building, Room 312, 300 Pasteur Drive, Stanford, CA 94305. nirk.quispe@stanford.edu (N.E.Q.C.) and rodolfo.vicetti@stanford.edu (R.D.V.M.).

Disclosures

The authors have no financial conflicts of interest.

mucosal permeability and susceptibility to genital pathogens while also implying that NET-EN generates less compromise of genital mucosal barrier function than DMPA.

Introduction

Depot-medroxyprogesterone acetate (DMPA) and norethisterone enanthate (NET-EN) are injectable progestins commonly used for contraception. For pregnancy prevention, women receive i.m. NET-EN or DMPA injections every 2 or 3 mo respectively. Although both provide effective contraception, there is concern that women using exogenous progestins are more likely to acquire HIV and other sexually transmitted infections. This public health dilemma has been long-standing and fueled by inconsistent results from the clinical studies used to examine this relationship (1–9). For the most part, however, these studies were not specifically designed to explore connections between HIV and progestin use, and methodological limitations likely contributed to variability in their results (10). These limitations included insufficient sample size, confounding of HIV incidence data by higher frequencies of unprotected sex in couples using hormonal contraception, imprecise knowledge of progestin use at time of HIV acquisition, and undefined estimates of HIV risk among women using different forms of progestins (11–13). For example, although one study found a 2-fold higher risk of male-to-female HIV transmission among women using injectable contraception, it did not delineate this risk between women using DMPA or NET-EN (14, 15). More recently, a randomized clinical trial that compared HIV acquisition in women using DMPA, levonorgestrel (LNG) implant, or copper intrauterine device reported that risk of HIV was not significantly increased in women randomized to any of these contraceptive choices (16). This study, however, did not include a group of women randomized to use no type of long-acting reversible contraceptive and could provide information only on the relative risk of HIV acquisition in women using DMPA, LNG implant, or a copper intrauterine device (16). It likewise did not include women randomized to initiate use of the injectable contraceptive NET-EN (16).

In addition to the obstacles that hinder clinical studies from defining precise relationships between HIV susceptibility and exogenous progestins, this public health controversy is fueled by the belief that putative connections lack biological plausibility (17). Addressing this long-standing concern using basic science and clinical research, the capacity of progestins to promote genital mucosal epithelial thinning and genital inflammation, alter the vaginal microbiota, suppress antiviral immunity, and enhance HIV replication have been identified as mechanisms with the potential to underlie this relationship (18–22). Alternatively, we showed that pharmacologically relevant serum levels of medroxyprogesterone acetate (MPA) in mice were associated with reduced genital expression of the cell–cell adhesion molecules desmoglein-1a (*Dsg1a*) and desmocollin-1 (*Dsc1*) (23). We likewise identified that DMPA-treated mice have increased genital mucosal permeability to low m.w. molecules and activated leukocytes, compromised genital mucosal barrier function, and enhanced susceptibility to genital HSV type 2 (HSV-2) and cell-associated HIV type 1 (HIV-1) infection (24). Guided by mouse model results, we discovered analogous changes in the expression of cell–cell adhesion molecules and mucosal barrier function in ectocervical tissue from women using DMPA or an LNG-releasing

intrauterine system (LNG-IUS) (24, 25). Based on these earlier results, in this study, we used a two-mouse model to explore the effects of NET-EN on genital mucosal barrier function and susceptibility to genital infection with HSV-2 or cell-associated HIV-1.

Materials And Methods

Mice, steroid treatments, and quantification of serum NET-EN levels

All procedures were approved by the Ohio State University Institutional Animal Care and Use Committee and the Stanford University Administrative Panel on Laboratory Animal Care. As indicated, 6- to 8-wk-old C57BL/6J (wild-type) and NOD-scid IL-2Rg^{null} (NSG) female mice were purchased from Jackson Laboratory (Bar Harbor, ME). As indicated, mice were s.c. injected for five consecutive days with 100 μ l of PBS alone or a PBS solution containing 1 mg of NET-EN (Sigma-Aldrich, Laramie, WY) (prepared by glass-to-glass homogenization and sonication), whereas other mice were s.c. administered a single dose of 1 mg of DMPA (Depo-Provera) or 2 mg of methylprednisolone (MePRDL) (Depo-Medrol) (both Pfizer, New York, NY). Where indicated, cells collected by vaginal lavage were stained with crystal violet to identify wild-type and NSG mice in the estrus stage of the estrous cycle (26). Also as indicated, mice were euthanized 24 h after the fifth NET-EN treatment, and the serum was sent to the Endocrine Technologies Support Core at the Oregon National Primate Research Center to quantify norethisterone (NET) levels by liquid chromatography–tandem triple quadrupole mass spectrometry (27).

RNA isolation and quantitative real-time PCR

Prior to storage at 280°C, excised vaginal tissue was placed in RNAlater (Qiagen, Hilden, Germany) for 24 h at 4°C. Using the manufacturer's instructions, RNA was isolated from stored tissues using the RNeasy Lipid Tissue Kit and RNase-free DNase Set (Qiagen). Samples were resuspended in nuclease-free water to quantify RNA using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) (samples routinely displayed 260/280 and 260/230 ratios > 1.8). Relative gene expression was calculated using 100 ng of RNA in the TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems, Foster City, CA) and the cycle threshold method with the following primers: glucocorticoid-induced leucine zipper (Mm00726417_s1), glucocorticoid receptor (GR) (Mm00433832_m1), arrestin β 2 (Mm00520666-g1), desmocollin-1 (Mm00496525_m1), desmoglein-1 α (Mm00809994_s1), kallikrein-related peptidase 7 (Mm01197332_g1), kallikrein-related peptidase 13 (Mm01197335_m1), kallikrein-related peptidase 14 (Mm00805093_m1), and pyruvate carboxylase (Mm00500992_m1) (the latter used to provide a housekeeping gene) (all primers are from Life Technologies, Pleasanton, CA). Quantitative real-time PCR (RT-qPCR) analyses were performed using the StepOne Real-Time PCR system (Applied Biosystems).

Immunofluorescence staining

As indicated, 5- to 10- μ m sections were obtained from formaldehyde-fixed and paraffin-embedded vaginal tissue and deparaffinized by the sequential immersion in 100% xylene, 100 and 96% ethanol, and sterile water. Ag retrieval was performed with sodium citrate buffer. Samples were incubated overnight at 4°C in PBT (i.e., PBS with 0.05% Tween 20

that contained 10% normal donkey serum; Abcam, Cambridge, MA) and for 1 h at room temperature with rabbit antidesmoglein-1 [clone EPR6766(B); Abcam] (1:200 dilution). Slides were washed and incubated 1 h at room temperature with Alexa Fluor 488–labeled donkey anti-rabbit IgG (Abcam) (1:500 dilution) and counter-stained with DAPI (23) for examination with a Confocal A1 Microscope (Nikon Instruments, Melville, NY) (all images were acquired using identical microscope settings). To quantify DSG1 protein levels, images were analyzed via ImageJ software as previously described (24, 28). All analyses were performed by investigators unaware of the treatment groups the mice had been assigned.

Genital permeability assays

As previously described, mice in estrus- and progesterin-treated mice were sedated and intravaginally (ivag) administered 10 μ l of PBS containing 50 μ g of Lucifer yellow CH lithium salt (molecular mass = 457.2 Da) and 62.5 μ g of dextran Texas Red (molecular mass = 70 kDa) (both Life Technologies) to assess vaginal permeability to low–molecular mass molecules (23–25). As already described, human PBMC (hPBMC) were activated and labeled with 5 μ M carboxyfluorescein succinimidyl ester (Cell-Trace CFSE; Life Technologies) to evaluate genital leukocyte permeability (25). Briefly, cells were resuspended in RPMI 1640 (10^8 cells per milliliter), and 10 μ l of this suspension was ivag administered to sedated animals. Mice were euthanized 15 h later for use in confocal microscopy studies that assessed hPBMC penetration into genital tissue. Using Image J software, hPBMC incursion into vaginal mucosa was defined by measuring the depth (micrometer) that CFSE-hPBMC penetrated between the epithelial apical and basal layers, whereas entry of cells into the vaginal lamina propria was defined by quantifying lamina propria fluorescence from CFSE-labeled hPBMC (pixels/ $10^4 \mu\text{m}^2$).

HSV infection

Using methods described previously (24), untreated mice in estrus or mice 1 d after their fifth NET-EN treatment or 5 d after a single DMPA treatment were sedated for ivag inoculation with 10^4 PFUs of HSV-2 333 in 10 μ l of RPMI 1640 (HSV-2 was kindly provided by Dr. R. L. Hendricks). HSV-induced pathology was evaluated daily via a 5-point scale: 0, no pathology; 1, mild vulvar erythema; 2, moderate vulvar erythema; 3, severe vulvar erythema; 4, perineal ulceration; and 5, extension of perineal ulceration to surrounding areas (29). Mice were euthanized when pathology scores ≥ 3 were detected or if they displayed encephalopathic changes. In other studies, identical groups of mice were ivag infected with 3×10^6 PFUs of HSV-1q–GFP in 10 μ l of RPMI 1640 as previously described(24) and euthanized 24 h later to assess fluorescent virus dissemination in vaginal tissue by confocal microscopy.

HIV infection

Using methods already described (24), NSG mice were engrafted with hPBMC (10^6 cells per mouse). Nine days after engraftment, mice remained untreated or were s.c. administered DMPA or NET-EN. Mice were sedated 14 d after hPBMC engraftment and ivag inoculated with 2×10^6 HIV-1–Ba-L–infected hPBMC in 10 μ l of RPMI 1640 (untreated mice in estrus at the time of infection provided controls). Mice were monitored daily, and those remaining alive 14 d postinfection were euthanized. In separate studies, identically engrafted and

progesterin-treated mice were euthanized 10 d after ivag inoculation with 10^6 HIV-1_{Ba-L}-infected hPBMC in 10 μ l of RPMI 1640. Plasma was obtained to measure HIV-1 RNA copies by Abbott RT-qPCR assay, and ex vivo splenocyte culture supernatants were used to detect infectious HIV-1 particles via TZM-BL luciferase assay (splenocyte culture supernatants from uninfected PBMC-engrafted mice and HIV-1_{Ba-L} aliquots provided negative and positive controls, respectively) (30–36).

Statistical considerations

Statistical analyses were performed using Prism 7 (GraphPad, La Jolla, CA). Normality was assessed using the D'Agostino and Pearson omnibus test or Shapiro–Wilk test (when sample numbers were ≥ 8). Depending on data distribution, unpaired Student t tests or unpaired Mann–Whitney *U* tests were used to compare two groups. For comparing more than two groups, one-way ANOVA with Tukey or Dunnett post hoc test or Kruskal–Wallis test with Dunn post hoc test were used (depending again on data distribution). Area under the curve analyses were used to compare HSV-induced pathology between untreated mice infected in estrus and NET-EN- or DMPA-treated mice. Survival was evaluated with Kaplan–Meier curves and log-rank tests used to compare cumulative survival incidence after genital infection with HSV-2 or cell-associated HIV-1. Any *p* values ≤ 0.05 were deemed statistically significant.

Results

Identifying an NET-EN dosing schedule that generates pharmacologically relevant serum levels of NET in mice

Because DMPA is the progesterin predominantly used in experimental models to promote genital pathogen susceptibility (37), we needed to first define an NET-EN dosing schedule that produces pharmacologically relevant serum levels in mice. Although initial work using RIAs indicated that peak serum NET levels in women after i.m. injection with 200 mg of NET-EN were 4–20 ng/ml (38, 39), more recent pharmacokinetic data generated with liquid chromatography–tandem mass spectrometry showed this dose generated peak serum NET concentrations that were closer to 35 ng/ml (40). Using established guidelines for converting drug doses between humans and laboratory animals (41), we created a dosing schedule for mice that was designed to achieve serum NET levels comparable to these clinical values. In these studies, we identified that daily administration of 1 mg of NET-EN for 5 d produced mean NET serum concentrations of 33 ng/ml, providing strong evidence that use of this dosing regimen in subsequent experiments would define genital tract changes induced by pharmacologically relevant serum concentrations of the drug (Fig. 1A).

NET-EN does not alter vaginal expression of glucocorticoid-regulated genes

Progesterin binding to intracellular steroid receptors is known to initiate events that alter gene expression (42). Whereas most progestins, including LNG and NET-EN, demonstrate high progesterone receptor (PR) affinity and low GR affinity, MPA is characterized by high PR affinity but greater GR affinity relative to other progestins (43–45). However, previous research from our laboratory demonstrated that vaginal tissue from mice treated with DMPA do not exhibit the signature changes in glucocorticoid-responsive gene expression seen in

mice administered the glucocorticoid MePRDL (24). Based on those results, we hypothesized that glucocorticoid-responsive gene expression is comparable in the vagina of NET-EN- and DMPA-treated mice but dissimilar from that found in mice treated with the pure GR agonist MePRDL. As posited, we found expression of multiple glucocorticoid-responsive genes, including glucocorticoid-induced leucine zipper (*Tsc22d3*), arrestin β 2 (*Arrb2*), and the GR (*Nr3c1*), was similar in vaginal tissue from mice in estrus and NET-EN- and DMPA-treated mice but significantly different from mice treated with MePRDL (Fig. 1B). These results were consistent with a prior report that NET-EN possesses negligible GR-binding affinity (45) and implied that changes in genital mucosal permeability or pathogen susceptibility elicited in NET-EN-treated mice would not be sequelae to NET-EN-mediated activation of the GR.

NET-EN treatment of mice enhances genital mucosal permeability

In earlier work, we identified that DMPA- and LNG-treated mice display significant increases in genital mucosal permeability compared with mice in the estrus stage of the estrous cycle (24). Extending these results in the current investigation, we showed that compared with estrus-stage mice, DMPA- and NET-EN- treated mice exhibit similar increases in genital mucosal permeability to low m.w. molecules (Fig. 2A). We also saw that entry of ivag administered activated human leukocytes into the vaginal epithelial tissue was facilitated by DMPA or NET-EN treatment (Fig. 2B, 2C). Although leukocyte incursion into more superficial layers of the vaginal epithelium was comparable in mice treated with DMPA or NET-EN, deeper penetration of these cells into the vaginal lamina propria was greater in DMPA- versus NET-EN-treated mice (Fig. 2B, 2D). Taken together, these studies established that treatment of mice with DMPA or NET-EN increases permeability of the genital mucosa, while also implying DMPA is more likely than NET-EN to facilitate leukocyte penetration into deeper layers of vaginal tissue.

NET-EN treatment significantly reduces cell-cell adhesion molecule expression in vaginal tissue

In prior publication, we reported that DMPA- and LNG-mediated increases in genital mucosal permeability were associated with reduced genital expression of the cell-cell adhesion molecules *Dsg1a* and *Dsc1* (24). Using similar methodology in the current study, we likewise found that compared with levels detected in estrus-stage mice, expression of the cell-cell adhesion molecule genes *Dsg1a* and *Dsc1* was significantly reduced in vaginal tissue from mice treated with DMPA or NET-EN (Fig. 3A). Although DSG1 protein levels were also significantly decreased in vaginal tissue from DMPA- and NET-EN-treated mice versus mice in estrus (Fig. 3B), more profound decreases in DSG1 protein were induced by DMPA treatment (Fig. 3C). To explore a potential mechanism for this differential decrease in DSG1 protein, we used vaginal tissue from DMPA- and NET-EN-treated mice to quantify the expression of kallikrein-related peptidases, serine proteases shown to cleave DSG1 protein (46, 47). Consistent with the reduced levels of DSG1 protein in vaginal tissue from DMPA-versus NET-EN-treated mice (Fig. 3C), we uncovered that DMPA treatment was associated with significantly higher levels of kallikrein-related peptidase 7 (*Klk7*), kallikrein-related peptidase 13 (*Klk13*), and kallikrein-related peptidase 14 (*Klk14*) (Fig. 3D). Taken together, these findings identified that DMPA and NET-EN similarly inhibited

vaginal Dsg1a and Dsc1 gene expression and also suggested that the reduced levels of DSG1 protein detected in DMPA-treated mice were secondary to DMPA-mediated increases in vaginal kallikrein-related peptidase expression.

NET-EN treatment promotes susceptibility of wild-type mouse to genital HSV-2 infection

Prior report showed that although vaginal epithelial thinning was comparable among mice in diestrus and DMPA-treated mice, only the latter group displayed significant increases in genital mucosal permeability and susceptibility to genital HSV-2 infection (24). Because our current investigation identified that DMPA- and NET-EN-treated mice display similar increases in genital mucosal permeability (Fig. 2), we hypothesized that NET-EN- and DMPA-treated mice are equally susceptible to genital HSV-2 infection. As posited, we detected widespread genital tissue dissemination of fluorescent HSV-1 in DMPA- and NET-EN-treated mice, but not in mice infected in estrus (Fig. 4A). Consistent with these results, ivag infection of estrus-stage wild-type mice with 10^4 PFUs of HSV-2 caused no morbidity or mortality, whereas infection of DMPA- and NET-EN-treated mice uniformly produced genital pathology and lethal encephalopathic changes (Fig. 4B–D). Although HSV-2 infection induced 100% mortality in mice treated with DMPA or NET-EN, more severe genital pathology and reduced survival times were generated by DMPA treatment (Fig. 4B–D).

NET-EN increases susceptibility of humanized mice to genital infection with cell-associated HIV-1

We concluded the current investigation by using hPBMC-engrafted NSG mice to compare the effects of DMPA or NET-EN treatment on susceptibility to ivag infection with cell-associated HIV-1_{Ba-L}. In initial studies, hPBMC-engrafted NSG mice (i.e., humanized mice) were untreated or administered DMPA or NET-EN. Fourteen days after hPBMC engraftment, untreated mice in estrus and mice treated with progestin were ivag inoculated with 2×10^6 HIV-1-infected hPBMC. Two weeks postinfection, we saw 100, 65, and 0% survival in untreated controls, NET-EN-treated mice, and DMPA-treated mice, respectively (Fig. 5A). In separate studies, identically engrafted and progestin-treated NSG mice were ivag infected with a smaller inoculum of 10^6 HIV-1-infected hPBMC. Euthanizing all mice 10 d postinfection, systemic HIV-1 infection was detected in 100% of mice treated with DMPA or NET-EN but in no mice infected in estrus. Compared with NET-EN-treated mice, however, DMPA-treated mice demonstrated significant increases in both infectious HIV-1-incultured splenocyte supernatants (Fig. 5B) and HIV-1 RNA copy numbers in plasma (Fig. 5C). Although these studies identified uniform susceptibility to ivag infection with cell-associated HIV-1_{Ba-L} in DMPA- and NET-EN-treated humanized mice, they also implied that DMPA is more likely than NET-EN to promote systemic spread of the virus after genital inoculation.

Discussion

The favorable impact of contraception on maternal health and life expectancy (48) does not mitigate the need to comprehensively define the effects of DMPA and other long-acting reversible contraceptives on antiviral immunity and HIV transmission efficiency. Among

currently available options, the relationship between DMPA and HIV susceptibility is the one most often examined in clinical investigation, and meta-analytic assessments of all available higher quality studies identified a 1.4-fold greater risk of HIV acquisition in women using DMPA versus no hormonal contraception (10). Although a relatively large number of studies were available for this risk estimate calculation, the efficiency of HIV transmission among women using LNG-IUS versus no hormonal contraception is mostly unexplored (49). Likewise, although no clinical study identified NET-EN as a significant HIV risk factor, relatively few of these studies were deemed of sufficient quality to be informative (10, 50). Moreover, among the five clinical studies considered suitably informative for inclusion in a meta-analytic review, four identified an increased but statistically nonsignificant risk of HIV acquisition among women using NET-EN versus no hormonal contraception (10, 50–55).

Because the effects of NET-EN have also been underexplored in experimental models of genital infection, we used the current study to compare genital mucosal barrier function and genital pathogen susceptibility in estrus-stage, DMPA-treated, and NET-EN-treated mice. These studies newly identify that mice with pharmacologically relevant serum levels of NET display significantly reduced genital expression of the cell–cell adhesion molecule DSG1, increased genital mucosal permeability, and increased susceptibility to genital HSV-2 and cell-associated HIV-1 infection. As mucosal barrier function is compromised in the genital tract of both DMPA- and NET-EN-treated mice, our findings further imply that these progestins share a biological effect with the capacity to promote genital pathogen transmission. Conversely, direct comparison of DMPA- and NET-EN-treated mice in the current investigation identifies DMPA as more likely to reduce vaginal DSG1 protein levels, facilitate deeper penetration of genital tissue by activated leukocytes, and promote systemic dissemination of genitally inoculated cell-associated HIV-1. The current investigation also reveals that treatment with DMPA is more likely than NET-EN to enhance genital tissue expression of kallikrein-related peptidases. As these proteases cleave cell–cell adhesion molecules, their increased expression in DMPA-treated mice may contribute to the lower levels of DSG1 protein and greater compromise of genital mucosal barrier function in these animals. However, alternative explanation exists for the differential effects of MPA and NET in the female mouse genitals. Prior research suggests NET has lower affinity for the PR than MPA (43, 56, 57), a characteristic of the drug that has potential to reduce *in vivo* NET potency. Likewise, unlike MPA, a portion of NET is aromatized *in vivo* to ethinyl estradiol, a potent estrogen with high estrogen receptor affinity (58). Because susceptibility of DMPA-treated humanized mice to genital infection with cell-associated HIV-1 is eliminated by concomitant treatment with exogenous estrogen (25), it is possible that aromatic conversion of NET to a more estrogenic compound *in vivo* makes NET-EN less likely than DMPA to enhance genital pathogen susceptibility.

Although defining precise mechanisms that cause greater compromise of antiviral immunity in the genital tract of DMPA-versus NET-EN-treated mice will require further investigation, our studies do establish that NET-EN treatment promotes mouse susceptibility to genital infection with HSV-2 and cell-associated HIV-1. Additional investigation will likewise be needed to resolve if NET-EN causes similar effects in women. Despite these limitations, it seems likely that current findings will inform interpretation of clinical data and help prioritize future clinical research. As analogous changes in genital DSG1 protein levels,

genital mucosal barrier function, and genital mucosal permeability are seen in DMPA- and LNG-treated mice and women using DMPA and LNG (23, 59), our findings also imply that similar changes may occur in the genital tract of women using NET-EN. Likewise, current evidence that treating mice with DMPA or NET-EN significantly increases susceptibility to genital HIV-1 infection intimates that even large-scale clinical studies may have a reduced capacity to resolve variable HIV transmission rates in women using DMPA, NET-EN, LNG-IUS, or other forms of unopposed progestin. Our work may also have relevance for data interpretation from studies where all participants are assigned to use some form of reversible contraception or when participants are randomized to contraceptive choices that have uncertain effects on HIV transmission. Although identifying the forms of long-acting reversible contraception least likely to promote HIV transmission remains both an important and daunting obligation, our findings provide a strong indication that these efforts will continue to be informed by exploration of murine and other appropriate experimental models.

Acknowledgments

We thank the Endocrine Technologies Core (Oregon National Primate Research Center) for the serum progestin levels that were quantified. HIV-1-Ba-L was obtained through the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (from Dr. Suzanne Gartner, Dr. Mikulas Popovic, and Dr. Robert Gallo).

This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (Awards R01HD094634 and R01HD072663), The Ohio State University College of Medicine, the Stanford University School of Medicine, and the Oregon National Primate Research Center (Award P51OD011092). The authors are solely responsible for content of this publication, which does not necessarily represent official National Institutes of Health views.

Abbreviations used in this article:

DMPA	depot-medroxyprogesterone acetate
GR	glucocorticoid receptor
HIV-1	HIV type 1; hPBMC, human PBMC
HSV-2	HSV type 2; ivag, intravaginal(ly)
LNG	levonorgestrel
LNG-IUS	LNG-releasing intrauterine system
MePRDL	methylprednisolone
MPA	medroxyprogesterone acetate
NET	norethisterone
NET-EN	norethisterone enanthate
NSG	NOD-scid IL-2Rg ^{null}
PR	progesterone receptor
RT-qPCR	quantitative real-time PCR

References

1. Bulterys M, Chao A, Habimana P, Dushimimana A, Nawrocki P, and Saah A. 1994 Incident HIV-1 infection in a cohort of young women in Butare, Rwanda. *AIDS* 8: 1585–1591. [PubMed: 7848595]
2. Martin HL Jr., Nyange PM, Richardson BA, Lavreys L, Mandaliya K, Jackson DJ, Ndinya-Achola JO, and Kreiss J. 1998 Hormonal contraception, sexually transmitted diseases, and risk of heterosexual transmission of human immunodeficiency virus type 1. *J. Infect. Dis* 178: 1053–1059. [PubMed: 9806034]
3. Kiddugavu M, Makumbi F, Wawer MJ, Serwadda D, Sewankambo NK, Wabwire-Mangen F, Lutalo T, Meehan M, Xianbin, and Gray RH; Rakai Project Study Group. 2003 Hormonal contraceptive use and HIV-1 infection in a population-based cohort in Rakai, Uganda. *AIDS* 17: 233–240. [PubMed: 12545084]
4. Lavreys L, Chohan V, Overbaugh J, Hassan W, McClelland RS, Kreiss J, Mandaliya K, Ndinya-Achola J, and Baeten JM. 2004 Hormonal contraception and risk of cervical infections among HIV-1-seropositive Kenyan women. *AIDS* 18: 2179–2184. [PubMed: 15577651]
5. Baeten JM, Benki S, Chohan V, Lavreys L, McClelland RS, Mandaliya K, Ndinya-Achola JO, Jaoko W, and Overbaugh J. 2007 Hormonal contraceptive use, herpes simplex virus infection, and risk of HIV-1 acquisition among Kenyan women. *AIDS* 21: 1771–1777. [PubMed: 17690576]
6. Morrison CS, Richardson BA, Mmro F, Chipato T, Celentano DD, Luoto J, Mugerwa R, Padian N, Rugepao S, Brown JM, et al.; Hormonal Contraception and the Risk of HIV Acquisition (HC-HIV) Study Group. 2007 Hormonal contraception and the risk of HIV acquisition. *AIDS* 21: 85–95. [PubMed: 17148972]
7. Reid SE, Dai JY, Wang J, Sicalwe BN, Akpomiemie G, Cowan FM, Delany-Moretlwe S, Baeten JM, Hughes JP, Wald A, and Celum C. 2010 Pregnancy, contraceptive use, and HIV acquisition in HPTN 039: relevance for HIV prevention trials among African women. *J. Acquir. Immune Defic. Syndr* 53: 606–613. [PubMed: 19838129]
8. Wand H, and Ramjee G. 2012 The effects of injectable hormonal contraceptives on HIV seroconversion and on sexually transmitted infections. *AIDS* 26: 375–380. [PubMed: 22156970]
9. Balkus JE, Brown ER, Hillier SL, Coletti A, Ramjee G, Mgodini N, Maman B, Reid C, Martinson F, Soto-Torres L, et al. 2016 Oral and injectable contraceptive use and HIV acquisition risk among women in four African countries: a secondary analysis of data from a microbicide trial. *Contraception* 93: 25–31. [PubMed: 26519646]
10. Polis CB, Curtis KM, Hannaford PC, Phillips SJ, Chipato T, Kiarie JN, Westreich DJ, and Steyn PS. 2016 An updated systematic review of epidemiological evidence on hormonal contraceptive methods and HIV acquisition in women. *AIDS* 30: 2665–2683. [PubMed: 27500670]
11. Morrison CS, and Nanda K. 2012 Hormonal contraception and HIV: an unanswered question. *Lancet Infect. Dis* 12: 2–3. [PubMed: 21975268]
12. Ralph LJ, McCoy SI, Shiu K, and Padian NS. 2015 Hormonal contraceptive use and women's risk of HIV acquisition: a meta-analysis of observational studies. *Lancet Infect. Dis* 15: 181–189. [PubMed: 25578825]
13. Gray RH 2012 Use of hormonal contraceptives and risk of HIV-1 transmission. *Lancet Infect. Dis* 12: 507; author reply 510–511.
14. Heffron R, Donnell D, Rees H, Celum C, Mugo N, Were E, de Bruyn G, Nakku-Joloba E, Ngunjiri K, Kiarie J, et al.; Partners in Prevention HSV/HIV Transmission Study Team. 2012 Use of hormonal contraceptives and risk of HIV-1 transmission: a prospective cohort study. [Published erratum appears in 2012 *Lancet Infect. Dis*. 12: 98.] *Lancet Infect. Dis* 12: 19–26. [PubMed: 21975269]
15. Beksinska ME, Smit J, and Kleinschmidt I. 2012 Use of hormonal contraceptives and risk of HIV-1 transmission. *Lancet Infect. Dis* 12: 509; author reply 510–511.
16. Evidence for Contraceptive Options and HIV Outcomes (ECHO) Trial Consortium. 2019 HIV incidence among women using intramuscular depot medroxyprogesterone acetate, a copper intrauterine device, or a levonorgestrel implant for contraception: a randomised, multicentre, open-label trial. [Published erratum appears in 2019 *Lancet* 394: 302] *Lancet* 394: 303–313.

17. Shelton JD 2012 Use of hormonal contraceptives and risk of HIV-1 transmission. *Lancet Infect. Dis* 12: 507–508; author reply 510–511.
18. Murphy K, Irvin SC, and Herold BC. 2014 Research gaps in defining the biological link between HIV risk and hormonal contraception. *Am. J. Reprod. Immunol* 72: 228–235. [PubMed: 24548147]
19. Ralph LJ, Gollub EL, and Jones HE. 2015 Hormonal contraceptive use and women's risk of HIV acquisition: priorities emerging from recent data. *Curr. Opin. Obstet. Gynecol* 27: 487–495. [PubMed: 26536211]
20. Huijbregts RP, Helton ES, Michel KG, Sabbaj S, Richter HE, Goepfert PA, and Hel Z. 2013 Hormonal contraception and HIV-1 infection: medroxyprogesterone acetate suppresses innate and adaptive immune mechanisms. *Endocrinology* 154: 1282–1295. [PubMed: 23354099]
21. Quispe Calla NE, Ghonime MG, Cherpes TL, and Vicetti Miguel RD. 2015 Medroxyprogesterone acetate impairs human dendritic cell activation and function. *Hum. Reprod* 30: 1169–1177. [PubMed: 25740884]
22. Ray RM, Maritz MF, Avenant C, Tomasicchio M, Dlamini S, van der Spuy Z, and Hapgood JP. 2019 The contraceptive medroxyprogesterone acetate, unlike norethisterone, directly increases R5 HIV-1 infection in human cervical explant tissue at physiologically relevant concentrations. *Sci. Rep* 9: 4334. [PubMed: 30867477]
23. Quispe Calla NE, Vicetti Miguel RD, Aceves KM, Torres A, and Cherpes TL. 2019 Depot-medroxyprogesterone acetate reduces genital cell-cell adhesion molecule expression and increases genital herpes simplex virus type 2 infection susceptibility in a dose-dependent fashion. *Contraception* 100: 397–401. [PubMed: 31302121]
24. Quispe Calla NE, Vicetti Miguel RD, Boyaka PN, Hall-Stoodley L, Kaur B, Trout W, Pavelko SD, and Cherpes TL. 2016 Medroxyprogesterone acetate and levonorgestrel increase genital mucosal permeability and enhance susceptibility to genital herpes simplex virus type 2 infection. *Mucosal Immunol.* 9: 1571–1583. [PubMed: 27007679]
25. Quispe Calla NE, Vicetti Miguel RD, Glick ME, Kwiek JJ, Gabriel JM, and Cherpes TL. 2018 Exogenous oestrogen inhibits genital transmission of cell-associated HIV-1 in DMPA-treated humanized mice. *J. Int. AIDS Soc* 21: e25063.
26. McLean AC, Valenzuela N, Fai S, and Bennett SA. 2012 Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J. Vis. Exp* 15: e4389.
27. Blue SW, Winchell AJ, Kaucher AV, Lieberman RA, Gilles CT, Pyra MN, Heffron R, Hou X, Coombs RW, Nanda K, et al. 2018 Simultaneous quantitation of multiple contraceptive hormones in human serum by LC-MS/MS. *Contraception* 97: 363–369. [PubMed: 29407362]
28. Schneider CA, Rasband WS, and Eliceiri KW. 2012 NIH image to ImageJ: 25 years of image analysis. *Nat. Methods* 9: 671–675. [PubMed: 22930834]
29. Cherpes TL, Harvey SAK, Phillips JM, Vicetti Miguel RD, Melan MA, Quispe Calla NE, and Hendricks RL. 2013 Use of transcriptional profiling to delineate the initial response of mice to intravaginal herpes simplex virus type 2 infection. *Viral Immunol.* 26: 172–179. [PubMed: 23638732]
30. Gartner S, Markovits P, Markovitz DM, Kaplan MH, Gallo RC, and Popovic M. 1986 The role of mononuclear phagocytes in HTLVIII/LAV infection. *Science* 233: 215–219. [PubMed: 3014648]
31. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, and Kabat D. 1998 Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol* 72: 2855–2864. [PubMed: 9525605]
32. Platt EJ, Bilska M, Kozak SL, Kabat D, and Montefiori DC. 2009 Evidence that ecotropic murine leukemia virus contamination in TZM-bl cells does not affect the outcome of neutralizing antibody assays with human immunodeficiency virus type 1. *J. Virol* 83: 8289–8292. [PubMed: 19474095]
33. Takeuchi Y, McClure MO, and Pizzato M. 2008 Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research. *J. Virol* 82: 12585–12588. [PubMed: 18842727]
34. Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, and Kappes JC. 2002 Emergence of resistant human immunodeficiency virus type 1 in patients receiving

- fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother* 46: 1896–1905. [PubMed: 12019106]
35. Derdeyn CA, Decker JM, Sfakianos JN, Wu X, O'Brien WA, Ratner L, Kappes JC, Shaw GM, and Hunter E. 2000 Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol* 74: 8358–8367. [PubMed: 10954535]
 36. Russell ES, Kwiek JJ, Keys J, Barton K, Mwapasa V, Montefiori DC, Meshnick SR, and Swanstrom R. 2011 The genetic bottleneck in vertical transmission of subtype C HIV-1 is not driven by selection of especially neutralization-resistant virus from the maternal viral population. *J. Virol* 85: 8253–8262. [PubMed: 21593171]
 37. Hall OJ, and Klein SL. 2017 Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. *Mucosal Immunol.* 10: 1097–1107. [PubMed: 28401937]
 38. Fotherby K, Howard G, Shrimanker K, Elder M, and Bye PG. 1978 Plasma levels of norethisterone after single and multiple injections of norethisterone oenanthate. *Contraception* 18: 1–6. [PubMed: 679682]
 39. Fotherby K, Towobola O, Muggeridge J, and Elder MG. 1983 Norethisterone levels in maternal serum and milk after intramuscular injection of norethisterone oenanthate as a contraceptive. *Contraception* 28: 405–411. [PubMed: 6673900]
 40. Friedrich C, Berse M, Klein S, Rohde B, and Hochel J. 2018 In vivo formation of ethinylestradiol after intramuscular administration of norethisterone enantate. *J. Clin. Pharmacol* 58: 781–789. [PubMed: 29522253]
 41. Nair AB, and Jacob S. 2016 A simple practice guide for dose conversion between animals and human. *J. Basic Clin. Pharm* 7: 27–31. [PubMed: 27057123]
 42. Louw-du Toit R, Perkins MS, Hapgood JP, and Africander D. 2017 Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy. *Biochem. Biophys. Res. Commun* 491: 140–146. [PubMed: 28711501]
 43. Kuhl H 2005 Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric* 8(Suppl. 1): 3–63.
 44. Hapgood JP, Kaushic C, and Hel Z. 2018 Hormonal contraception and HIV-1 acquisition: biological mechanisms. *Endocr. Rev* 39: 36–78. [PubMed: 29309550]
 45. Winneker RC, Bitran D, and Zhang Z. 2003 The preclinical biology of a new potent and selective progestin: trimegestone. *Steroids* 68: 915–920. [PubMed: 14667983]
 46. Shaw JL, Petraki C, Watson C, Bocking A, and Diamandis EP. 2008 Role of tissue kallikrein-related peptidases in cervical mucus remodeling and host defense. *Biol. Chem* 389: 1513–1522. [PubMed: 18844451]
 47. Muytjens CM, Vasiliou SK, Oikonomopoulou K, Prassas I, and Diamandis EP. 2016 Putative functions of tissue kallikrein-related peptidases in vaginal fluid. *Nat. Rev. Urol* 13: 596–607. [PubMed: 27603220]
 48. World Health Organization. Hormonal contraceptive eligibility for women at high risk of HIV: guidance statement. Geneva: : WHO Available at: https://www.who.int/reproductivehealth/publications/family_planning/HC-and-HIV-2017/en/.
 49. Vicetti Miguel RD, Quispe Calla NE, and Cherpes TL. 2018 Levonorgestrel and female genital tract immunity: time for a closer look. *J. Infect. Dis* 218: 1517–1518. [PubMed: 29917111]
 50. Polis CB, Phillips SJ, Curtis KM, Westreich DJ, Steyn PS, Raymond E, Hannaford P, and Turner AN. 2014 Hormonal contraceptive methods and risk of HIV acquisition in women: a systematic review of epidemiological evidence. *Contraception* 90: 360–390. [PubMed: 25183264]
 51. Kleinschmidt I, Rees H, Delany S, Smith D, Dinat N, Nkala B, and McIntyre JA. 2007 Injectable progestin contraceptive use and risk of HIV infection in a South African family planning cohort. *Contraception* 75: 461–467. [PubMed: 17519153]
 52. Myer L, Denny L, Wright TC, and Kuhn L. 2007 Prospective study of hormonal contraception and women's risk of HIV infection in South Africa. *Int. J. Epidemiol* 36: 166–174. [PubMed: 17175547]

53. Morrison CS, Skoler-Karppoff S, Kwok C, Chen PL, van de Wijgert J, Gehret-Plagianos M, Patel S, Ahmed K, Ramjee G, Friedland B, and Lahteenmaki P. 2012 Hormonal contraception and the risk of HIV acquisition among women in South Africa. *AIDS* 26: 497–504. [PubMed: 22156973]
54. McCoy SI, Zheng W, Montgomery ET, Blanchard K, van der Straten A, de Bruyn G, and Padian NS. 2013 Oral and injectable contraception use and risk of HIV acquisition among women in sub-Saharan Africa. *AIDS* 27: 1001–1009. [PubMed: 23698064]
55. Crook AM, Ford D, Gafos M, Hayes R, Kamali A, Kapiga S, Nunn A, Chisembele M, Ramjee G, Rees H, and McCormack S. 2014 Injectable and oral contraceptives and risk of HIV acquisition in women: an analysis of data from the MDP301 trial. *Hum. Reprod* 29: 1810–1817. [PubMed: 24838704]
56. Philibert D, Bouchoux F, Degryse M, Lecaque D, Petit F, and Gaillard M. 1999 The pharmacological profile of a novel norpregnane progestin (trimegestone). *Gynecol. Endocrinol* 13: 316–326. [PubMed: 10599548]
57. Heffron R, Achilles SL, Dorflinger LJ, Hapgood JP, Kiarie J, Polis CB, and Steyn PS. 2019 Pharmacokinetic, biologic and epidemiologic differences in MPA- and NET-based progestin-only injectable contraceptives relative to the potential impact on HIV acquisition in women. [Published erratum appears in 2019 *Contraception* 100: 88.] *Contraception* 99: 199–204. [PubMed: 30576636]
58. Chu MC, Zhang X, Gentschein E, Stanczyk FZ, and Lobo RA. 2007 Formation of ethinyl estradiol in women during treatment with norethindrone acetate. *J. Clin. Endocrinol. Metab* 92: 2205–2207. [PubMed: 17341557]
59. Quispe Calla NE, Vicetti Miguel RD, Trout W, and Cherpes TL. 2017 HIV and hormonal contraception: bench and bedside. *J. Acquir. Immune Defic. Syndr* 74: e85–e86. [PubMed: 27599006]

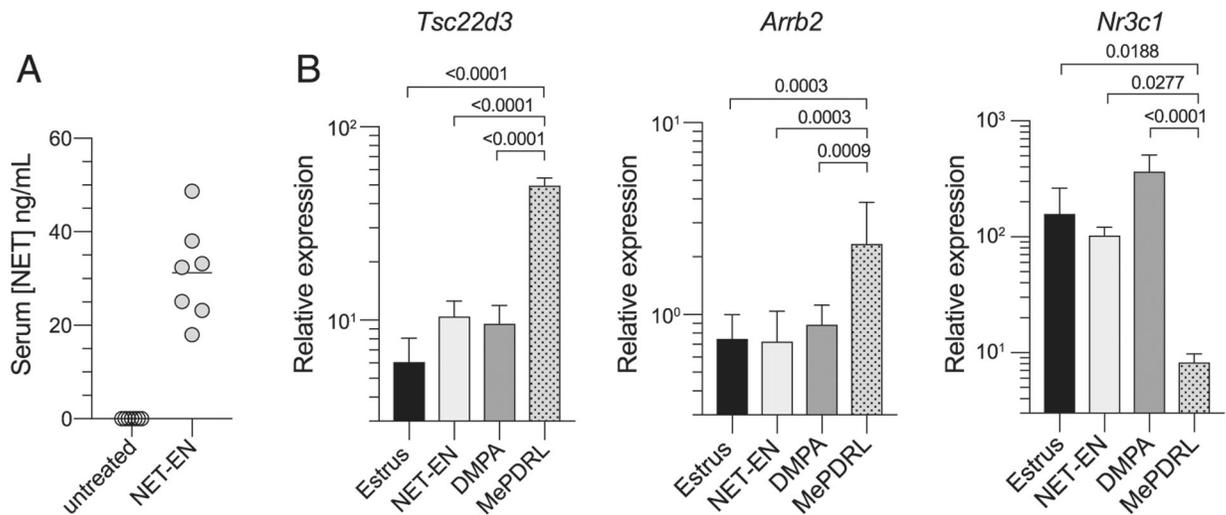


FIGURE 1. Vaginal tissue from NET-EN- and MePRDL-treated mice exhibit dissimilar expression of GR-regulated genes.

(A) Scatter plots comparing NET serum levels in untreated mice in estrus versus NET-EN-treated mice indicate the NET-EN dosing schedule devised for this study produced pharmacologically relevant drug concentrations. (B) Comparing vaginal expression of the GR-regulated gene glucocorticoid-induced leucine zipper (*Tsc22d3*), arrestin β 2 (*Arrb2*), and GR (*Nr3c1*) in mice in estrus and DMPA-, NET-EN-, or MePRDL-treated mice shows markedly different gene expression in mice administered an exogenous progestin versus the pure GR agonist MePRDL. Data displayed are pooled from two independent experiments with comparable results (seven animals per group); one-way ANOVA and Dunnett post hoc test provided statistical comparison; bars denote mean $6 \pm$ SD.

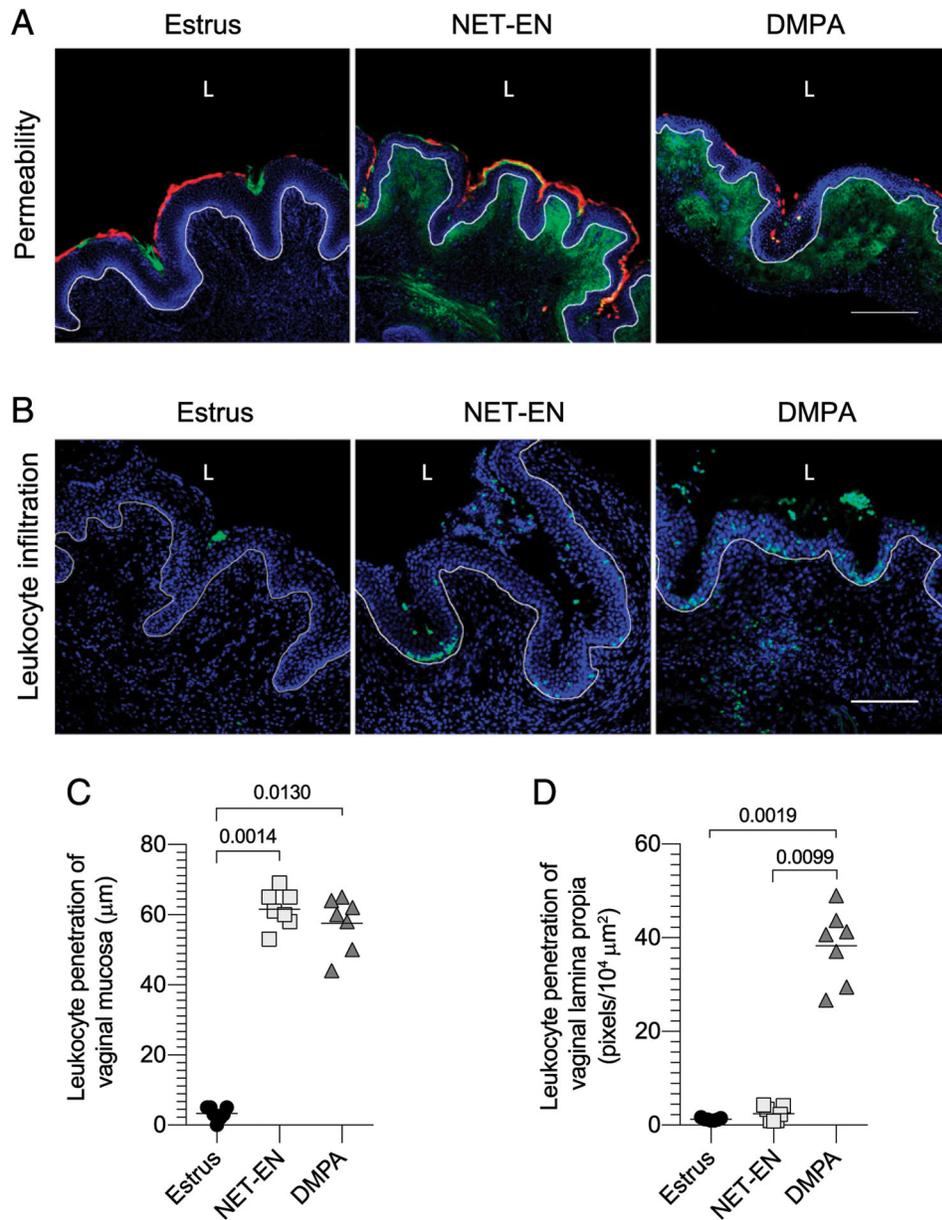


FIGURE 2. DMPA and NET-EN increase genital mucosal permeability.

(A) Representative confocal images of vaginal tissue from wild-type mice in estrus or treated with NET-EN or DMPA show these progestins increased tissue permeability to the low-molecular weight fluorescent molecule Lucifer yellow (LY) (457 Da) (green) but not the higher molecular mass dextran conjugated with Texas Red (70 kDa) (red); DAPI (blue). Scale bar, 200 µm. White lines drawn to delimit vaginal mucosal versus lamina propria tissue. (B–D) Identically treated mice were used to compare the entry of activated hPBMC into vaginal mucosal and lamina propria tissue. (B) Representative images typify the enhanced entry of CFSE-labeled hPBMC (green) into vaginal tissue of DMPA- and NET-EN-treated mice; DAPI blue. Scale bar, 100 µm. Drawn white lines demarcate mucosa from lamina propria. (C) Image analyses show that DMPA and NET-EN comparably increased

hPBMC entry into vaginal epithelium. (D) Conversely, there was significantly greater hPBMC incursion into the lamina propria of DMPA- versus NET-EN-treated mice (as defined using ImageJ to calculate pixels per $10^4 \mu\text{m}^2$ of CFSE-labeled hPBMC). Data in (C) and (D) are pooled from two independent experiments in which similar results were obtained (seven animals per group). Statistical analyses were performed using one-way ANOVA and Dunnett post hoc test (bars denote mean \pm SD). L, lumen.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

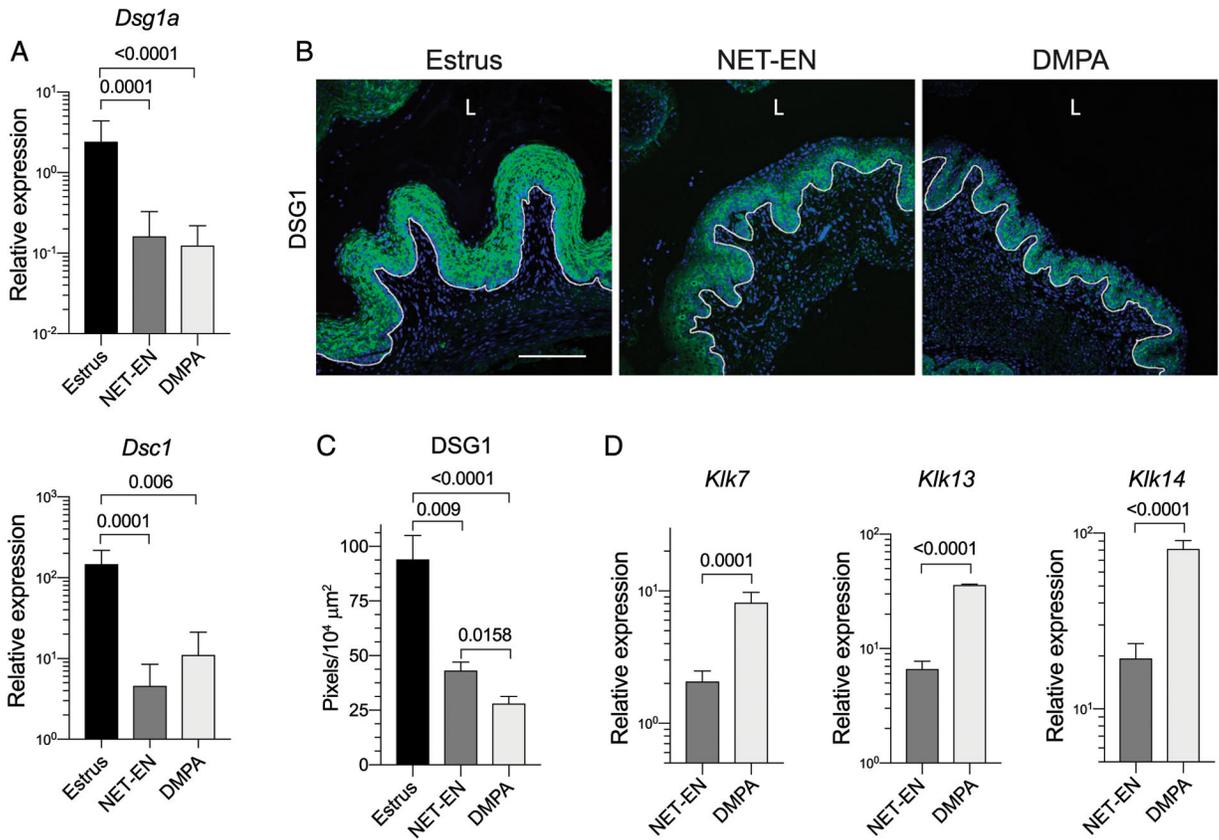


FIGURE 3. Treatment of mice with DMPA or NET-EN reduces vaginal DSG1 levels. (A) Comparing vaginal *Dsg1a* and *Dsc1* gene expression in mice in estrus versus DMPA- and NET-EN-treated mice shows significantly lower levels of these cell–cell adhesion molecules in progestin-treated mice; one-way ANOVA and Dunnett’s post hoc tests provided statistical analyses; bars denote mean ± SD. (B) These groups were also used in DSG1 protein studies, and representative images typify lower levels of DSG1 in DMPA- and NET-EN-treated mice; DSG1a (green); DAPI (blue). Scale bar, 100 μm. (C) Image analyses show both progestins significantly reduced DSG1, but DMPA caused greater reduction; one-way ANOVA and Dunnett post hoc test were used for statistical analyses; bars indicate mean ± SD. (D) Significantly greater kallikrein-related 7, 13, and 14 levels were detected in vaginal tissue from DMPA- versus NET-EN–treated mice; bars denote mean ± SD; unpaired Student t tests used for statistical analyses. Data in (A), (C), and (D) were pooled from two independent studies with similar results (five to eight mice per group). *Dsc1*, desmocollin; *Dsg1a*, desmoglein-1α; L, lumen.

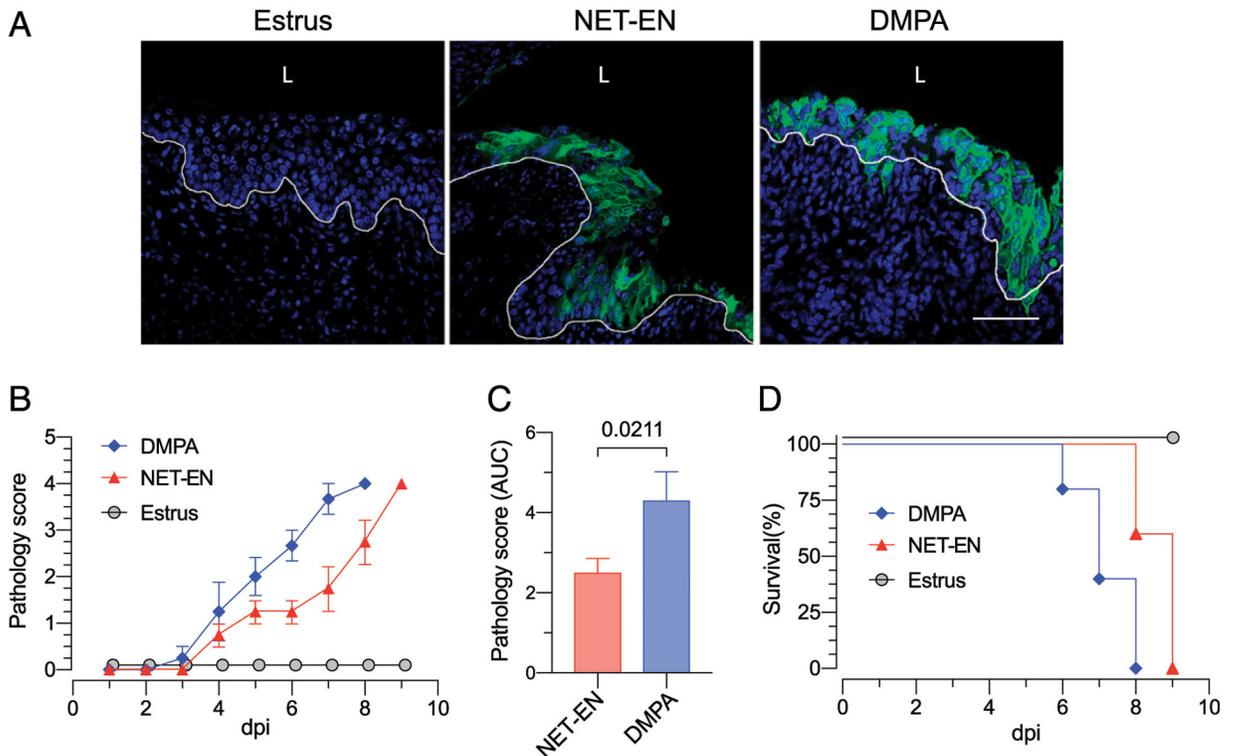


FIGURE 4. DMPA and NET-EN treatment increases mouse susceptibility to genital HSV-2 infection.

(A) Wild-type mice in estrus and mice treated with DMPA or NET-EN were euthanized 24 h after ivag infection with 3×10^6 PFUs of HSV-1q-GFP, and representative images illustrate widespread dissemination of virus only in vaginal tissue from progestin-treated mice. Scale bar, 50 μm. DAPI (blue); HSV-1q-GFP (green). (B–D) In these panels, identically treated groups of mice were used to assess morbidity and mortality generated by ivag infection with 10^4 PFUs of HSV-2 333. (B) All progestin-treated mice developed genital pathology, but (C) HSV-2 infection induced greater pathology in DMPA- versus NET-EN-treated mice; AUC were calculated for individual mice treated with DMPA or NET-EN and compared using unpaired t test. (D) Survival curves depict the 100% mortality induced by genital HSV-2 infection of DMPA- and NET-EN-treated mice and that no mice infected in estrus succumbed; data representative of two independent studies with comparable results (five mice per group in each study); log-rank tests compared cumulative survival incidence after HSV-2 infection (estrus versus DMPA or NET-EN, $p < 0.0001$) (DMPA versus NET-EN, $p = 0.018$). AUC, area under the curve; HSV-1q-GFP, HSV-1-expressing green-fluorescent protein; L, lumen.

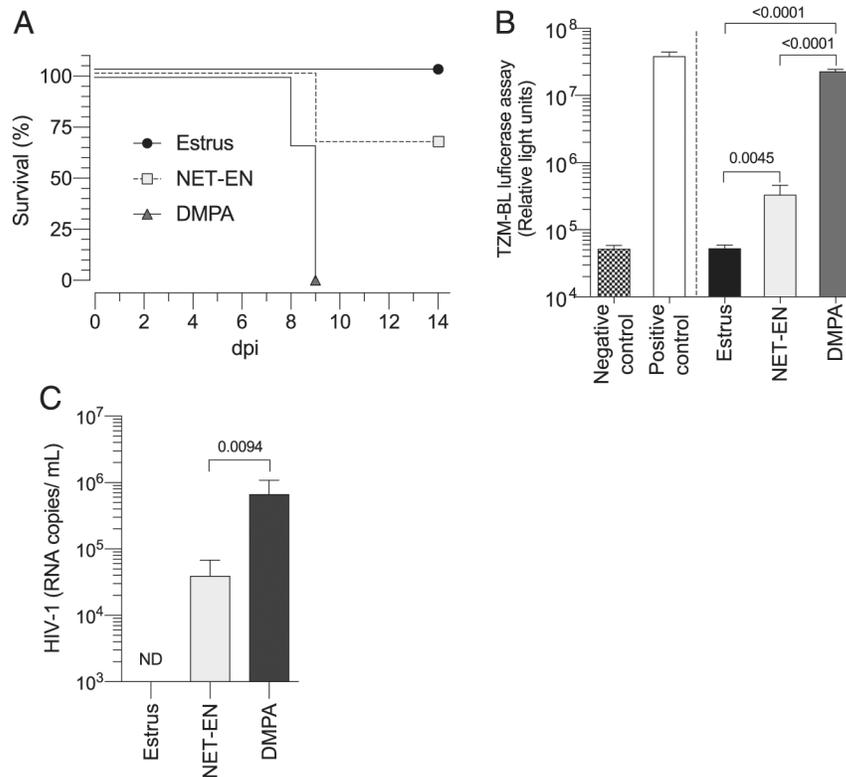


FIGURE 5. Treatment of humanized mouse with DMPA or NET-EN increases susceptibility to genital infection with cell-associated HIV-1.

(A) Estrus-stage, DMPA-treated, and NET-EN-treated hPBMC-NSG (i.e., humanized) mice were monitored daily after ivag infection with 2×10^6 HIV-1_{Ba-L}-infected hPBMC. Although all DMPA-treated mice succumbed to infection by 9 days postinfection (dpi), ~65% of the NET-EN-treated mice were alive at 14 dpi. (B and C) Other identically treated humanized mice were ivag inoculated with 10^6 HIV-1-infected hPBMC and euthanized at 10 dpi to assess systemic HIV-1 infection and viral load. (B) Quantitative BTZM-bl luciferase assays show higher HIV levels detected in splenocyte cultures from DMPA- versus NET-EN-treated mice (splenocytes from uninfected mice and media-diluted HIV-1_{Ba-L} were used as positive and negative controls, respectively). (C) RT-qPCR assays found significantly higher HIV-1 RNA copy number in plasma of DMPA- versus NET-EN-treated mice; data depicted is representative of two independent experiments that produced similar results (three mice per group) (bars denote mean \pm SD). Statistical analyses were performed with one-way ANOVA and Kruskal-Wallis post hoc test (B) or unpaired Mann-Whitney *U* test (C); log rank tests were used to compare cumulative survival incidence after HIV-1 infection (estrus versus DMPA, $p = 0.001$; estrus versus NET-EN, $p = 0.138$).