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Reproductive tract immune cells from pregnant women or those using depot medroxyprogesterone acetate show no excess susceptibility to HIV-1: results of an *ex vivo* fusion assay

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Abstract

Introduction—*Ex vivo* fusion assays offer an efficient method for studying HIV-1 entry associated with contraceptive use and pregnancy outside of cohort studies of HIV-1 incidence.

Methods—We measured *ex vivo* HIV-1 fusion to cervical or endometrial immune cells from 3 groups of women: pregnant, non-pregnant not using hormonal or intrauterine contraception, and using depot medroxyprogesterone acetate (DMPA).

Results and Conclusions—There was no excess susceptibility to HIV-1 fusion of cells from pregnant women or DMPA users compared to controls. Although the number of target cells in endometrium was higher in DMPA users compared to controls, HIV-1 fusion was lower.

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Implications—In *ex vivo* assays, HIV-1 showed no enhanced fusion to cervical immune cells from pregnant women or DMPA users compared to controls, and lower fusion to endometrial immune cells from DMPA users. This assay is useful for studying hormonal and contraceptive effects on HIV-1 entry into reproductive tract immune cells.

Introduction

Seventy-one million women worldwide used injectable hormonal contraception in 2019, and this method continues to be the most prevalent contraceptive used in regions of Africa with persistently high HIV incidence [1] [2] [3]; therefore the potential effects of contraceptives on risk of HIV acquisition is of great public health significance. Depot medroxyprogesterone acetate (DMPA) is a commonly used injectable contraceptive. Observational studies suggested HIV acquisition risk may increase with DMPA use: two meta-analyses reported that women using DMPA face a 40–50% greater risk of acquiring HIV [2] [4]. The ECHO study, a randomized trial evaluating HIV-1 acquisition in women using DMPA, copper intrauterine devices, and levonorgestrel implants, showed no significant difference in HIV-1 incidence by arm [5], prompting the World Health Organization to affirm the safety of progestin-based contraceptive methods without restriction for women at heightened risk of HIV acquisition [6]. However, HIV-1 incidence was high in all ECHO arms, and pregnancy incidence rates ranged from 1.8 to 3.3 per 100 person-years in intention-to-treat analyses, highlighting the need for integrated HIV-1 and reproductive health counseling.

Meta-analyses and modeling studies identify pregnancy as a time of increased HIV-1 vulnerability [7, 8]. Biological factors such as hormonal status and/or changes in sexual behavioral during pregnancy could contribute to these findings. Comprehensive reproductive and sexual health counseling requires discussion of HIV-1 risk related not only to use of various contraceptive methods, but also to use of no method, and to pregnancy. In this cross-sectional study, we used a novel *ex vivo* HIV-1 fusion assay [9] to determine whether there are differences in HIV-1 susceptibility of target cells isolated from cervix and endometrium from women using DMPA or during pregnancy compared to non-pregnant women not using hormonal or intrauterine contraception. This approach allows for identification of biological factors associated with HIV-1 entry into target cells while limiting the variability from other risk factors such as sexual behaviour.

Methods

The University of California San Francisco approved the study protocol and consent materials; all procedures were performed in accordance with university regulations. Cisgender HIV-negative women 18–45 years of age were recruited from the San Francisco Bay Area; detailed description of the recruitment, screening and sample collection for the control group are published [10]. We recruited DMPA participants from on-line ads and family planning clinics, and pregnant women at 20–22 weeks of gestation at the time of their appointment at an abortion clinic; we selected the 20–22 week window to capture a high progesterone state.

Participants were eligible if they were not on immuno-modulating medications, not breast-feeding, and willing to refrain from vaginal intercourse and intravaginal product use for 3 days prior to sample collection. For the control group, we included women with regular menstrual cycles every 21–35 days and who were not using hormonal or intrauterine contraception [10]. For the DMPA group, women had to be using DMPA for at least 3 months. Screening labs included blood test for HIV-1 and urine tests for pregnancy (except in the pregnant group), *Chlamydia trachomatis* and *Neisseria gonorrheae*. We extensively pre-screened participants for eligibility by phone in the control and DMPA groups and there were no screening failures. We directly recruited pregnant participants in the abortion clinic and 45% agreed to participate.

We instructed women in the control group how to measure urine luteinizing hormone and scheduled the sample collection visit 7–11 days after a positive test to coincide with peak progesterone levels in cycling women. We collected samples in the DMPA group 2–4 weeks after injection (verified by the clinic), timed to coincide with peak medroxyprogesterone acetate levels. Prior to sample collection, we tested urine for pregnancy (except in the pregnant group) and checked vaginal pH (as a marker of possible bacterial vaginosis). We used a 3 mm biopsy cannula for the endometrial biopsy and a Tischler biopsy forceps for the cervical biopsy targeted to the transformation zone (the junction of Lugol's staining and non-staining epithelium), or at the cervical os if the ectocervix was entirely Lugol's staining. In pregnant participants, we collected cervical biopsies at time of dilator placement prior to the abortion; we did not collect endometrial biopsies due to concerns about contamination from pregnancy tissue. We measured plasma progesterone from all participants at the time of sample collection to verify ovulation in the control group, suppression in the DMPA group, and pregnancy-related levels in the pregnant group.

Samples delivered to the lab on ice within 2–3 hours of collection underwent enzymatic digestion to generate single cell suspensions, which we then stored in aliquots at −80°C. We have previously published detailed methods for the HIV-1 fusion assay [10]. Briefly, the HIV-1 fusion virus contains a β-lactamase–Vpr chimeric protein that is delivered into the cytoplasm of target cells after fusion and can be detected by fluorescence flow cytometry based on cleavage of a fluorescent substrate by β-lactamase. The HIV-1 fusion virus ZM247Fv2 used in these experiments is a heterosexually transmitted founder virus originally isolated from a Zambian woman [11] that was the most fusogenic clone of 37 isolates studied in endometrial cells [10]. We incubated thawed single cell suspensions with the fusion virus for 1.5 hours, added the fluorescent substrate CCF2, incubated overnight to allow substrate cleavage, and then incubated with an immunophenotyping antibody panel to quantitate expression levels of various cell surface markers [10]. Flow cytometry was performed on a FACS Aria II, and compensation and manual gating were performed on Flow Jo Version X. The flow cytometric analysis of the HIV fusion data allowed measurement of the numbers of total and fused immune (CD45+) cells of 5 relatively abundant HIV-1 target cell types defined as follows: macrophages (CD14+ HLA-DR+ CD163+), Langerhans cells (CD207+), dendritic cells (CD1a+), tissue-resident memory CD4+ T cells (CD3+ CD4+ CD45RO+ CD69+) and 'other'.

We used descriptive statistics and Kruskal-Wallis or chi-square tests to compare demographic and clinical characteristics of participants by study group. To account for samples from 2 anatomic sites at one time point per woman, we used site-stratified generalized estimating equation (GEE) modeling with a log-binomial distribution to estimate mean proportions of fused immune cells (i) overall and by risk group, adjusted for age and (ii) by phenotype, within groups. Estimated 95% confidence intervals were based on robust standard errors and GEE score tests were used to assess statistically significant variation among groups. Statistical analyses were conducted using SAS version 9.4.

Results

Demographic and biological characteristics of study participants are shown in Table 1. The control group was significantly older and more likely to be college-educated (Table 1). Progesterone levels were significantly lower in the DMPA group and higher in pregnancy, as expected. In the control group, 2 participants had progesterone levels <0.5 ng/ml, indicating failure to ovulate; the next lowest value was 4.9, indicating that the rest of the participants (15/17, 88%) had ovulated.

For cervical immune cells, the mean total yield of live CD45+ immune cells was 3261.3 (range 46–11544); when compared by exposure group, the mean yield was relatively low in pregnant women (1032.8, range 176–2323) compared to controls (3725.3, range 46–8962) and DMPA users (3958.3, range 520–11544), but the variation among groups was not statistically significant (Kruskal-Wallis p=0.25).

Overall, 8.5% (95%CI 6.2%−11.4%) of cervical immune cells fused to HIV-1. The majority (7.1%) were CD4+ T cells, whereas fewer than 1% were macrophages, Langerhans, or dendritic cells (Figure 1A). There was no statistically significant association of the proportion of fused cells with age (p=0.73) or with exposure group. By group, the age-adjusted cervical fusion rate ranged from 9.6% (7.6–12.0%) in unexposed controls, to 8.3% (4.2–16.2%) in DMPA users (15% mean reduction versus controls), to 7.7% (4.3–13.8%) in pregnant women (22% mean reduction) (p=0.78).

One DMPA user tested positive for *Chlamydia trachomatis*. Since cervicitis is thought to increase HIV-1 susceptibility [12], we compared the percentages of fused cells in her cervical and endometrial samples to those from other DMPA users and found her results typical within both analyses. Accordingly, we retained this participant in the study.

For endometrial immune cells, the mean total yield of live CD45+ immune cells was 1825.0 (range 493–5779). The mean yield was lower in controls (1283.2, range 493–2625) than in DMPA users (3342.2, range 2011–5779) and the difference between groups was statistically significant (Kruskal-Wallis p=0.006).

For endometrial immune cells, 9.7% (95%CI, 8.4%−11.3%) overall fused to HIV-1. The majority were CD4+ T cells (6.2%) and macrophages (2.6%) (Figure 1B). The proportion of fused endometrial cells did not vary by age (p=0.78). The age-adjusted fusion rate in controls was 14.1% (11.5–17.1%) and in DMPA users was 6.6% (5.0–8.7%) (75% mean reduction) (p=0.01).

Discussion

Results of this study show no excess susceptibility to HIV-1 fusion of cervical or endometrial immune cells from pregnant women or DMPA users compared to controls, and are congruent with the *in vivo* findings of the ECHO trial showing no obvious harm signal of DMPA compared to 2 other contraceptive methods [5]. Our results indicate that although the number of HIV-1 target cells was higher in the endometrium of DMPA users than in controls, susceptibility to HIV-1 entry was lower. Our finding of enhanced HIV-1 susceptibility of macrophages from endometrium compared to cervix in DMPA users and controls expands previous observations that endometrial macrophages appear to have a unique susceptibility [10, 13]. Our results highlight the need for further study of the effects of factors known to alter endometrial biology, such as pregnancy and hormonal contraceptives, on HIV-1 entry into endometrial immune cells.

A previous studies of the female reproductive tract with the HIV-1 fusion assay used endocervical cells to characterize the phenotype of T cells susceptible to HIV entry; however that study provided no information about hormonal variables that might impact the findings such as time in the menstrual cycle or contraceptive use [14]. Our previous work assessed HIV-1 fusion to cervical and endometrial cells from women using copper or levonorgestrel intrauterine devices or combined oral contraceptives, and showed no enhanced fusion in these groups compared to the control group samples collected in the luteal phase [10]. The present study extends those studies to effects of other hormonal states, specifically DMPA and pregnancy, on HIV-1 entry.

Strengths of our study include consistent findings from distinct anatomical sites and the inclusion of pregnant women, demonstrating the logistical value of *ex vivo* studies for assessing the effects of hormonal status on biological parameters such as HIV entry. Limitations of the study are the small sample sizes of the DMPA and pregnant groups, and the lack of samples from later in pregnancy and postpartum, when HIV-1 susceptibility appears to be highest [7]. To date, results from this assay have not been directly correlated with an *in vivo* prospective study of HIV-1 acquisition. However, we previously showed that exposure of endometrial CD4+ T cells in this assay to semen-derived amyloid fibrils, which are known to increase HIV-1 infection [15], resulted in increased fusion [16], indicating that the assay can detect biologically relevant factors that alter HIV-1 entry.

Adoption of *ex vivo* assays to triangulate with epidemiologic and *in vivo* data may enhance our collective ability to identify biological correlates of HIV entry by anatomic site, such as contraceptive use and hormonal levels associated with pregnancy, facilitating the long-term goal of integration of comprehensive reproductive options counseling with HIV-1 counseling for women.

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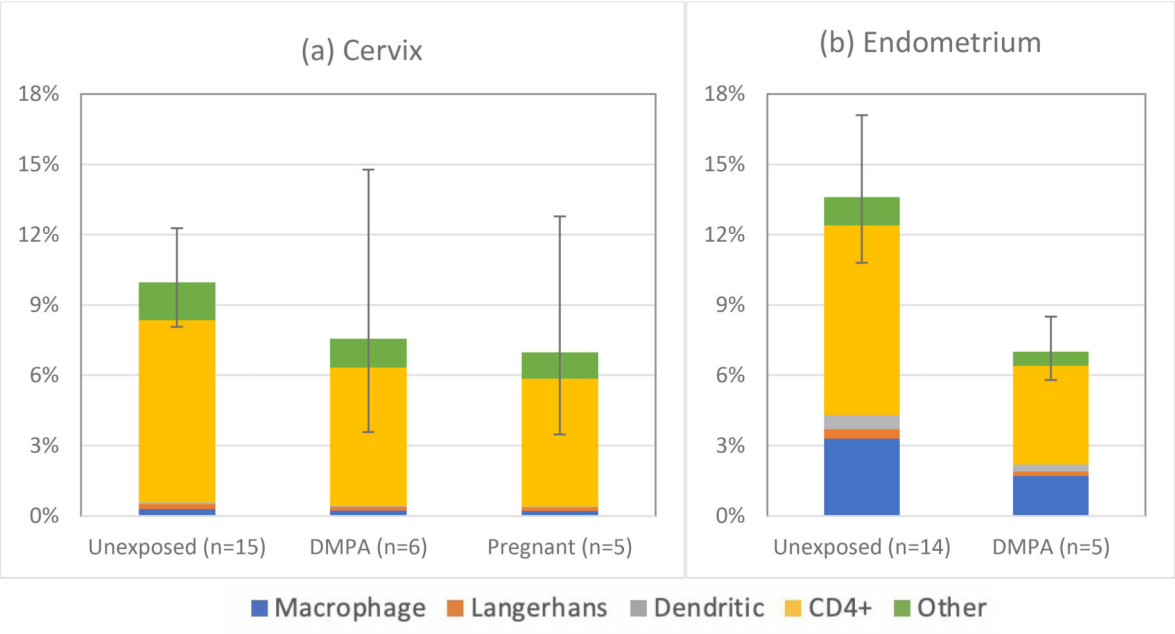

Figure 1. Fusion assay combined with immunostaining to identify and quantify phenotypes of immune cells that support HIV-1 fusion in (a) cervix and (b) endometrium.

Bar graphs depict the mean (95% CI) prevalence of immune cells (live CD45+ cells) supporting ZM247v2 fusion (overall height of the bar) by participant contraception group. Contributions of four major cellular subsets to the overall prevalence are identified by color clade. Endometrial biopsies were not collected from pregnant women.

Table 1:

Demographic and biologic characteristics of study participants

	Control N=17	Pregnant N=5	DMPA N=6	P value
Age: median (min,max)	32 (21,46)	25 (20,44)	23.5 (21,26)	0.014 *
BMI: median (min,max)	26 (20,56)	24 (18,27)	25 (21,33)	
Education: n(%)				0.49 *
Some high school	2 (12)	0 (0)	1 (17)	
High school	0 (0)	1 (20)	2 (33)	0.028 ¶
Some college	5 (29)	4 (80)	3 (50)	
Completed college	10 (59)	0 (0)	0 (0)	
Race: n(%)				
White	5 (29)	4 (80)	3 (50)	
Black/AA	9 (53)	1 (20)	1 (17)	0.29 ¶
Mixed	0	0	1 (17)	
Other	3 (18)	0	1 (16)	
Ethnicity: n(%)				
Latina	5 (29)	1 (20.0)	5 (83)	
Non-Latina	12 (71)	4 (80.0)	1 (17)	0.47 ¶
Partner #: median (min,max)	8 (1,50)	5 (2,20)	2.5 (1,5)	
Gravidity: median (min,max)	0 (0,7)	3 (1,5)	1 (0,2)	0.10 *
Smoker: n(%)				0.10 *
No	13 (76.5)	4 (80.0)	6 (100.0)	
Yes	4 (23.5)	1 (20.0)	0 (0.0)	0.44 ¶
Progesterone ng/ml: median (min,max)	9.5 (0.5,19) §	83 (58,101)	0.5 (0.5,0.6)	0.001 *

* Kruskal-Wallis test

¶ Chi square test