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Depot Medroxyprogesterone Acetate Administration Alters Immune Markers for HIV Preference and Increases Susceptibility of Peripheral CD4+ T Cells to HIV Infection

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ABSTRACT
Depot medroxyprogesterone acetate (Depo-Provera) has been associated with an increased risk of HIV acquisition. In a longitudinal study, we investigated the impact of Depo-Provera use by healthy women on expression of immune markers for HIV preference and on HIV infection ex vivo at baseline (visit 1), 1 mo (visit 2), and 3 mo (visit 3) after Depo-Provera treatment. We found a significant increase in the frequency and expression of integrin α4β7 on CD4+ T cells at visit 2. Interestingly, Hispanic but not black women exhibited a significant increase in integrin α4β7 cell numbers and expression levels at visit 2, whereas black but not Hispanic women exhibited a significant change in CCR5 and CD38 expression levels between visit 2 and visit 3. The frequency of terminal effector memory CD4+ T cells was decreased significantly in black women from visit 1 to visit 3. Virus production following ex vivo HIV infection of PBMCs was increased at visit 3 compared with visit 1. In black women, the frequency of HIV p24+CD4+ T cells was higher at visit 3 than at visit 1. Expression of integrin α4β7 on HIV p24+CD4+ T cells following ex vivo infection at visit 2 was significantly less than at visit 1. These results demonstrate that Depo-Provera alters the immune profile of peripheral CD4+ T cells and increases susceptibility to HIV infection ex vivo. The observation that these effects differed between women of different ethnicities has implications for developing effective and targeted strategies for HIV prevention.

INTRODUCTION
More than 210 million women worldwide used hormonal contraception in 2015 (1). For decades, a putative, adverse impact of hormonal contraception on HIV acquisition and transmission has been a concern, but results on this subject have remained controversial (2–12). Several studies indicate that use of depot medroxyprogesterone acetate (MPA) (Depo-Provera), an injectable form of hormonal contraceptive used by 35 million women globally, is associated with heightened risk of HIV acquisition and transmission (4, 6, 7, 9, 11, 13, 14). Two recent meta-analyses of multiple studies with stringent inclusion criteria indicate an increased risk of HIV infection in Depo-Provera users with an adjusted hazard ratio of 1.4–1.5 compared with subjects not using hormonal contraception (15, 16). Furthermore, an updated meta-analysis by Polis et al. (17) analyzing high-power
DEPO-PROVERA INCREASES HIV PREFERENCE MARKERS AND INFECTION

Studies published between 2014 and 2016 found a hazard ratio of 1.4 in women using Depo-Provera, compared with nonhormonal contraceptive controls. Despite these findings, Wall and colleagues (18, 19) report Depo-Provera use resulted in no increased risk of HIV acquisition among HIV-negative women with their HIV-positive partners or among HIV-positive women with HIV-negative men in Zambian discordant couples. The possibility that hormonal contraceptive use increases the risk of HIV acquisition has profound implications for family-planning policies, particularly in countries with high rates of HIV transmission. Thus, understanding how Depo-Provera modulates immune responses and affects HIV infection is critical for developing strategies for HIV prevention as well as for shaping policies for preventing unintended pregnancy.

Depo-Provera is known to modulate immune responses that can impact HIV susceptibility or infection (reviewed in Ref. 9). The synthetic progestin MPA has a high affinity for the progesterone receptor but has low affinity interactions with other steroid receptors including glucocorticoid, androgen, and mineralocorticoid receptors (20–23). MPA is thought to exhibit an immunosuppressive effect through its interaction with the glucocorticoid receptor (24). MPA but not progesterone (P4) suppresses cytokine production in TCR-stimulated PBMCs and in TLR-stimulated plasmacytoid dendritic cells (pDCs) (25, 26). MPA blocks downregulation of CXCR4 and CCR5 in PBMCs in response to TCR activation (25, 26). HIV replication is increased by MPA in activated CD8-depleted PBMCs or CD4+ T cells in vitro (25, 26). In contrast to MPA, P4 has been reported to downregulate CXCR4 and CCR5 in PBMCs in vitro, which is associated with reduced susceptibility to HIV (27). These results indicate that MPA and P4 mediate distinct immune functions despite the fact that both bind the progesterone receptor, and illustrate the complexity of the role of hormonal contraceptives in immune regulation.

The immunomodulatory effects of Depo-Provera on peripheral blood cells in women has been studied to a limited extent. One cross-sectional study showed no significant difference in the percentage of CCR5+CD8+ or CCR5+CD4+ T cells, but found a significant increase in expression (mean fluorescence intensity, MFI) of CCR5 on CD4+ T cells of Depo-Provera users compared with women not using hormonal contraception (28). TLR-9 stimulation of pDCs resulted in reduced production of TNF-α and IFN-α in Depo-Provera users compared with women not using hormonal contraception (29). Depo-Provera users also had decreased plasma levels of IFN-α and IL-8 but no change in T cell subsets compared with nonhormonal contraception users (29). In HIV-infected women on cART (median age of 32) no significant changes in cell-mediated immunity or plasma cytokine levels were observed except for a small, albeit significant, decrease in TGF-β (30). A significant decrease in the frequency of CD4+ CD25+ cells and a nearly significant decrease in CD8+CD38+ HLA DR+ cells were found in this population 3 mo after Depo-Provera injection, whereas an increased frequency of CD8+ Foxp3 (regulatory T) cells was observed 4 wk after Depo-Provera administration (30).

In this study, we have examined changes in immunological markers associated with preferential HIV infection of CD4+ T cells, including integrin α4β7, CCR5 (HIV coreceptor), and CD38 (activation), and have characterized CD4+ T cell subsets in PBMCs from women before (visit 1), 1 mo following (visit 2), and 3 mo following (visit 3) Depo-Provera injection. The 3 mo schedule was chosen because serum or plasma MPA concentrations are known to decrease over time and return to near baseline 3 mo after Depo-Provera injection (31, 32). At the time of each visit, we also assessed the susceptibility of freshly isolated PBMCs to HIV infection ex vivo, and measured HIV production and immunological markers on HIV p24+ cells. Our findings indicated that Depo-Provera treatment modified immune profiles of CD4+ T cells and increased HIV infection ex vivo. Of possible importance for clinical treatment of patients, we also observed that changes in the immune response and susceptibility to HIV infection following Depo-Provera injection were influenced by ethnicity and duration of treatment.

MATERIALS AND METHODS

Study design
These studies were approved by Rutgers, New Jersey Medical School Institutional Review Board. Participants were women receiving healthcare at Rutgers, New Jersey Medical School clinics located in Newark, NJ. Women who desired to use Depo-Provera, met the primary screening criteria as described below, and were willing to sign the informed consent form were recruited and compensated. The enrollment eligibility criteria were: 1) healthy, nonpregnant women aged 18–35; 2) no known or suspected HIV infection; 3) no use of hormonal contraception in the previous 2 mo; 4) negative for Chlamydia, gonorrhea, syphilis, genital herpes, bacterial vaginosis, trichomoniasis, presence of condyloma acuminata, carcinoma in situ of the cervix, or invasive cervical cancer; 5) no known or suspected immunosuppressive illness or condition; and 6) no sexual intercourse for 3 d prior to the enrollment visit. Eligibility was ascertained at the enrollment visit. Potential participants not fulfilling all inclusion criteria were excluded.

Blood was collected before Depo-Provera injection (visit 1), 1 mo following (visit 2), and 3 mo following the initial Depo-Provera injection (visit 3). PBMCs were isolated immediately after blood collection by Histopaque-1077 gradient centrifugation. Immune phenotypes of freshly isolated PBMCs were determined after isolation. PBMCs were exposed to HIV and maintained in RPMI 1640 with 10% heat-inactivated FBS and IL-2 (50 IU/ml). IL-2 was supplemented to cell cultures every 2 d.

Reagents
Histopaque-1077, FBS, human AB serum, RPMI 1640 medium, and PBS were from Sigma-Aldrich. Human IL-2 was from R&D Systems. V500-conjugated mouse IgG1e anti-human CD45 (clone HI30), Alexa Fluor 700-conjugated mouse IgG2a anti-human CD197 (CCR7) (clone 150503), allophycocyanin-Cy7–conjugated
Biotechnology. Integrin Ag (clone KC57) was from Beckman Coulter. FITC-conjugated PE-conjugated mouse IgG1 anti-controls in wash buffer were used for various cell surface markers or their appropriate isotype controls. HIV-1 p24 positive cells, after cell-surface marker staining, were analyzed using Flow cytometry. Live cells were selected for analysis. Following live/dead staining, cells were blocked in wash buffer (PBS, 2% FBS, 1% human AB serum) for 20 min at 4°C. Cells were stained with fluorochrome-conjugated Abs against various cell surface markers or their appropriate isotype controls in wash buffer for 20 min at 4°C. Cells were washed and fixed with 2% paraformaldehyde in PBS. For analysis of HIV-1 p24 positive cells, after cell-surface marker staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) for 20 min at 4°C. Cells were washed in BD Perm/Wash buffer (BD Biosciences) and stained with HIV-1 p24 Ab or isotype control Ab for 30 min at 4°C in BD Perm/Wash buffer. Cells were washed and fixed with 2% paraformaldehyde in PBS. Samples were analyzed using a BD LSR II (BD Biosciences). Results were analyzed with FlowJo (Tree Star).

During analysis lymphocytes were selected based on forward scatter versus side scatter. Dead cells were excluded from analysis. CD4+ T cells were identified as CD3+ and CD4+. Within the CD4+ T cell population, expression of surface markers integrin α4β7, CCR5, CD38, CCR7, CD45RA, and intracellular p24 were analyzed as described previously (33).

**Statistical analysis**

Wilcoxon matched-pairs signed rank test was used to compare values between study visits. Specifically, this method analyzed the difference in immune parameters or HIV infection within the same individual at different visits. Mann–Whitney U test was used to compare differences between uninfected and cells exposed to HIV-1BaL. Spearman correlation test was used to compare HIV infection (p24 levels or the p24+ cells) and surface marker expression at each visit. Statistical analysis of the immune phenotypes and correlation analysis was performed using GraphPad Prism version 7.0b (GraphPad Software). Regression analysis of log-transformed data of HIV p24 production was conducted using generalized estimating equations to account for the longitudinal nature of data collected from samples at baseline, 1, and 3 mo after treatment. The generalized estimating equation model of log 10–transformed p24 values was implemented with an autoregressive correlation structure that assumes correlations between observations on the same subject decline with time (34). Reanalysis of the data using other correlation structures (e.g., exchangeable) produced similar results.

**RESULTS**

**Subject characteristics**

A total of 39 healthy subjects meeting the eligibility criteria were enrolled in the study. Among these, 29 participants returned for all visits, six participants returned only for visit 2, and four participants returned only for visit 3. As expected based on the demographic characteristics of the recruitment clinical site in Newark, all subjects were black or Hispanic. Median age was 26 [interquartile range (IQR), 21.5–29.5]; 15 women were Hispanic (median age of 25, IQR 22–31), and 24 women were black (median age of 26.5, IQR 21.75–28.25). None of the subjects used any form of hormonal contraception including Depo-Provera for up to 10 mo before the time of recruitment.

**Expression of integrin α4β7, CCR5, and CD38 on peripheral CD4+ T cells from women pre- and post–Depo-Provera injection**

We first determined whether Depo-Provera injection resulted in changes in the cell surface expression of integrin α4β7, CCR5, and CD38, markers that are associated with preference for HIV infection in the CD4+ T cell population. The gating strategy is shown in Supplemental Fig. 1. Participant peripheral blood CD4+ T cells were analyzed before (visit 1), 1 mo following (visit 2), and 3 mo following (visit 3) Depo-Provera injection. There was an increase in the frequency of integrin α4β7+CD4+ T cells at visit 2 (median 49.7%, IQR 42.37–56.8%) compared with baseline (visit 1, median 44.15%, IQR 33.47–54.55%) (Fig. 1A, left). There was no significant difference in the frequency of integrin α4β7+CD4+ T cells between visit 2 and visit 3 (visit 3, median 42.95%, IQR 32.37–51.95%).
Differential immune phenotypes in blacks and Hispanics in response to Depo-Provera

Interestingly, we observed subtle differences in immune phenotypes between black and Hispanic women following Depo-Provera injection. In black women, there was a nonsignificant increase in the frequency and intensity of integrin α4β7 at visit 2 (median 51.3%, IQR 43.55–55.8%; median MFI 1636, IQR 1408.6–1771.25) compared with visit 1 (median 45.6%, IQR 40.2–53.7%; median MFI 1382, IQR 1202–1659.5) (Fig. 2A). There was a decline in the frequency and intensity of integrin α4β7 at visit 3 (median 42.24%, IQR 34.15–49.55%; median MFI 1303, IQR 1123–1472) compared with visit 2. We observed similar patterns of CCR5 expression on CD4+ T cells from black women following Depo-Provera injection compared with all donors combined, with an increase in the frequency and intensity of these markers at visit 2 (median 9.33%, IQR 5.45–15.15%; median MFI 1550, IQR 1487–1659.5) (Fig. 2B). There was a slight but nonsignificant increase in the frequency of CD38 expression at visit 3 (median 7.88%, IQR 5.84–11.2%; median MFI 1382, IQR 1202–1659.5) (Fig. 2C). There was a slight but nonsignificant increase in the frequency and intensity of integrin α4β7 at visit 2 (median 51.3%, IQR 43.55–55.8%; median MFI 1636, IQR 1408.6–1771.25) compared with visit 1 (median 45.6%, IQR 40.2–53.7%; median MFI 1382, IQR 1202–1659.5) (Fig. 1B, left). A similar increase in CCR5 expression from visit 1 to visit 2 was observed (visit 1, median 79.5, IQR 61.67–99.32; visit 2, median 101.8, IQR 78.2–131.24) (Fig. 1B, right). There was a measurable but nonsignificant decrease in the frequency of CCR5 on CD4+ T cells at visit 3 (median 7.88%, IQR 6.84–11.2%) compared with visit 2. We found a significant decrease in CCR5 MFI at visit 3 (median 83.3, IQR 62.5–94.7) compared with visit 2 (median 101.8, IQR 78.2–131.24) but not compared with visit 1.

There was no difference in the frequency of CD38+CD4+ T cells between visit 1 (median 47.6%, IQR 40.05–54.4%) and visit 2 (median 46.7%, IQR 37.1–57.4%). An observable but nonsignificant increase in intensity of CD38 expression on CD4+ T cells was found at visit 2 (median 2272, IQR 1553–2857) compared with visit 1 (median 1987, IQR 1577.5–2574.5) (Fig. 1C). There was a slight but nonsignificant increase in the frequency of CD38+CD4+ T cells at visit 3 (median 49.3%, IQR 37.1–53.6%) compared with visit 2. There was a significant decrease in the intensity of CD38 expression at visit 3 (median 1906, IQR 1423–2323.25) compared with visit 2. There was no significant difference in either frequency or intensity of CD38 at visit 3 compared with visit 1.

The frequency of CCR5+CD4+ T cells at visit 2 (median 9.33%, IQR 7.03–12.98%) was slightly, albeit not significantly, increased compared with baseline (visit 1, median 8.24%, IQR 6.69–11.55%) (Fig. 1B, left). A similar increase in CCR5 expression from visit 1 to visit 2 was observed (visit 1, median 79.5, IQR 61.67–99.32; visit 2, median 101.8, IQR 78.2–131.24) (Fig. 1B, right). There was a measurable but nonsignificant decrease in the frequency of CCR5 on CD4+ T cells at visit 3 (median 7.88%, IQR 6.84–11.2%) compared with visit 2. We found a significant decrease in CCR5 MFI at visit 3 (median 83.3, IQR 62.5–94.7) compared with visit 2 (median 101.8, IQR 78.2–131.24) but not compared with visit 1.

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or between visit 1 and visit 3. Results of analysis of MFI indicated a significant increase in expression of integrin α4β7 on CD4+ T cells at visit 2 (median 1554, IQR 1278–1718) compared with baseline (visit 1, median 1363, IQR 1136.5–1504.5; Fig. 1A, right). At visit 3, there was a significant decrease in the expression of integrin α4β7 on CD4+ T cells (median 1290, IQR 988–1514) compared with visit 2, but there was no significant difference in the expression of integrin α4β7 at visit 3 compared with visit 1.

FIGURE 1. Depo-Provera altered the expression of cell surface markers associated with HIV susceptibility.

PBMCs from subjects before and after Depo-Provera injection were isolated and the percentage and MFI of integrin α4β7 (A), HIV coreceptor CCR5 (B), and activation marker CD38 (C) were analyzed within the CD3+CD4+ T cell population by flow cytometry. Wilcoxon matched-pairs signed rank test was used to compare values from before Depo-Provera (visit 1), 1 mo after Depo-Provera (visit 2), and 3 mo after Depo-Provera (visit 3). Each dot represents one donor. Bars represent median and IQR. A p value ≥ 0.05 was considered significant, p > 0.05 was not significant (ns).
The percentage and MFI of cell surface markers integrin α4β7, CCR5, and CD38 on CD3+CD4+ T cells from freshly isolated PBMCs was analyzed within patients who identified as either black (A) or Hispanic (B) by flow cytometry. Each dot represents one donor. Bars represent median and IQR. Wilcoxon matched-pairs signed rank test was used for analysis. A p value ≤ 0.05 was considered significant, p > 0.05 was not significant (ns).
7.64–11.7%) and an increase in the CM subset between visit 2 and visit 3 (visit 1, median 16.9%, IQR 13.65–21.95%; visit 2, median 17.4%, IQR 14.4–21.3%; visit 3, median 20.7%, IQR 14.45–26.93%).

The percentage of TEM cells in black participants significantly decreased from visit 1 (13.65% median, IQR 10.32–13.5%) to visit 2 (12.2% median, IQR 7.98–11.65%) and from visit 1 to visit 3 (10.5% median, IQR 7.94–10.4%) (Fig. 3B). There was a noticeable nonsignificant increase in the naive and CM subsets in black women (naive visit 1, median 30%, IQR 20.3–44%; visit 2, median 37.3%, IQR 24.35–44.63%; visit 3, median 40.35%, IQR 22.93–43.13%) (CM visit 1, median 16.3%, IQR 12.6–23.6%; visit 2, median 17.05%, IQR 14.98–21.4%; visit 3, median 20.6%, IQR 14.8–27.28%). Analysis of the Hispanic patient group showed no
significant change in CD4+ T cell subsets between visits despite a nonsignificant decrease in the frequency of TEM cells after Depo-Provera injection (TEM visit 1, median 13%, IQR 7.72–19.85%; visit 2, median 10.85%, IQR 7.07–15.08%; visit 3, median 9.47%, IQR 7.54–11.48%) (Fig. 3C).

**Impact of Depo-Provera on susceptibility of PBMCs to HIV-1 ex vivo**

To determine whether Depo-Provera increased HIV infection of PBMCs ex vivo, freshly isolated PBMCs were exposed to HIV-1BaL at MOI 0.01. HIV production was determined by measuring HIV p24 levels in cell culture medium, as well as by flow cytometric analysis of the frequency and intensity of CD4+ T cells expressing HIV p24. There was a significant increase in HIV production in PBMCs at visit 3 compared with visit 1 (visit 1, median 1736.97 pg/ml, IQR 953.79–4950.55 pg/ml; visit 2, median 3190.95 pg/ml, IQR 980.97–8382.31; and visit 3, median 3683.52 pg/ml, IQR 1780.62–8459.79 pg/ml) (Fig. 4A). There was no significant difference in HIV p24 levels at day 7 between different visits among either black or Hispanic participants (Fig. 4B, 4C).

The number of infected CD4+ T cells and the intensity of HIV p24 staining measured on day 10 after HIV infection was lower, albeit nonsignificantly, at visit 2 than at visit 1 or visit 3 (visit 1, median 6.92%, IQR 3.82–13.8%, MFI median 967.5, IQR 640.75–1557.5; visit 2, median 3.75%, IQR 2.54–12.2%, MFI median 779, IQR 586–1136; visit 3, median 7.16%, IQR 5.06–14.5%, MFI median 915, IQR 785–1401) (Fig. 5A). The slight decrease in HIV p24 cells at visit 2 was not associated with an increase in cell death (Supplemental Fig. 2).

We found a significant increase in the frequency of HIV p24+ cells in black subjects at visit 3 (median 11.17% median, IQR 5.03–17.02%) compared with visit 1 (median 6.64%, IQR 3.68–10.4%) (Fig. 5B). The pattern of the HIV p24+ cell frequency among different visits in Hispanic women was comparable to black women, but unlike the results in black women, the differences were not significant (Fig. 5C). There was no difference in the intensity of HIV p24 expression among three visits in Hispanic women. These findings suggest that ethnicity plays a role in immune modulation in response to Depo-Provera that subsequently impacts HIV susceptibility.

**Effect of Depo-Provera on immunological markers for HIV preference**

We next determined whether Depo-Provera altered markers for HIV preference and CD4+ T cell subsets in HIV-infected cells. Prior to Depo-Provera injection (visit 1), the HIV p24+ population had a significantly higher percentage of integrin α4β7 (57.7% median, IQR 45.2–66.6%) compared with the p24− population in HIV-infected cells (46.7% median, IQR 38.1–50.3%) or uninfected cells (49% median, IQR 38.1–57.8%) (Fig. 6A). The HIV p24+ cell population also had a higher percentage of CCR5+ cells at visit 1 (35% median, IQR 22.8–53.8%) compared with the p24− population (14.8% median, IQR 9.17–27.1%) or uninfected cells (25.9% median, IQR 14.7–38.2%) (Fig. 6B). The frequency of CD38+ cells was also higher in the p24+ population (95% median, IQR 90.75–97.2%) than in the p24− cells (81.5% median, IQR 73.65–84.45%) or uninfected cells (85.2% median, IQR 76.5–87.4%) (Fig. 6C). The HIV p24+ population was mainly found in the EM subset (63.9% median, IQR 57.9–74.8%) followed by the CM subset (18.9% median, IQR 14.8–26.1%). TEM cells comprised 7.56% of p24− cells (median, IQR 5.48–12.3%) and naive cells were the least abundant (median 4.68%, IQR 2.52–8.71%) (Fig. 6D).

Similar profiles of HIV preference were observed in PBMCs after Depo-Provera treatment at visit 2 and at visit 3. The HIV p24− cell population had a higher frequency of cells expressing integrin α4β7, CCR5, and CD38 (Fig. 6) than the HIV p24+ population in infected or uninfected PBMCs.
Analysis of markers for HIV preference and CD4+ T cell subsets within HIV p24+ cells from PBMCs at different visits revealed a slight, but not significant decrease in the frequency of integrin α4β7 on CD4+ T cells at visit 2 (median 47.06%, IQR 37.35–58.5%) and visit 3 (median 49%, IQR 36–64.95%) compared with visit 1 (median 55.7%, IQR 45.2–66.6%) (Fig. 7A). The intensity of integrin α4β7 in HIV p24+CD4+ T cells was significantly reduced from visit 1 (median 4062, IQR 2992.5–5421.5) to visit 2 (median 2470, IQR 1876.75–4248.25). There was a slight increase in CCR5+ cells in HIV p24+ cells after Depo-Provera treatment (visit 1, median 35%, IQR 22.8–53.8%; visit 2, median 40.65%; IQR 25.92–55.6%; visit 3, median 42.1%, 30.35–52.95%) although the difference was not significant (Fig. 7B). There was no significant change in CD38+ cells within the HIV p24+ population among different visits (visit 1, median 95%, IQR 90.75–97.2%; visit 2, median 94.15%, IQR 90.45–97.13%; visit 3, median 95%, IQR 90.9–97.85%) (Fig. 7C). Analysis of CD4+ T cell subsets within the HIV p24+ population indicated a notable increase in the percentage of CM cells at visit 2 (median 25.25%, IQR 13.67–33.47%) compared with visit 1 (median 18.9%, IQR 14.8–26.1%). The frequency of EM cells was noticeably reduced in the p24+ population at visit 2 (median 58% median, IQR 48.27–73.32%) compared with visit 1 (median 63.9% median, IQR 57.8–74.8%) although the changes in HIV preferred CD4+ T cell subsets in response to Depo-Provera did not reach significance (Fig. 7D). Taken together, our results indicated that, whereas CD4+ T cells expressing integrin α4β7, CCR5 or CD38 were preferentially infected by HIV before and after Depo-Provera treatment, the level of integrin α4β7 in HIV-infected cells was significantly decreased at visit 2.

**DISCUSSION**

Our longitudinal study indicated that Depo-Provera administration increased the numbers of peripheral CD4+ T cells with markers for HIV preference and promoted HIV infection of PBMCs ex vivo. We found a significant increase in the percentage of integrin α4β7+CD4+ T cells and in the expression level of integrin α4β7 in monocytes (visit 2) after injection of Depo-Provera, when serum levels of MPA are highest (31, 32). There was also an increasing trend of CCR5 and CD38 expression at visit 2 compared with visit 1. The percentage and intensity of α4β7, CCR5, and CD38 decreased from visit 2 to visit 3 to levels comparable to visit 1, suggesting the immunomodulatory effects of Depo-Provera may depend on the serum MPA concentration (visit 2 > visit 3 > visit 1). There were differential changes in these markers depending on the race and ethnicity. There was a decreasing trend of TEM frequency from visit 1 to visit 3, and the reduction of TEM cells in black women was highly significant. Depo-Provera did not appear to change HIV preference significantly as HIV p24+ cells had higher levels of integrin α4β7, CCR5, and CD38 than HIV p24− cells regardless of Depo-Provera treatment. Interestingly, the expression level of integrin α4β7 on HIV p24+ cells was significantly lower at visit 2 compared with visit 1, and we also observed a nonsignificant decrease in the frequency of HIV p24− cells expressing integrin α4β7 from visit 1 to visit 3 (Fig. 7). We found that HIV production from PBMCs at visit 3 was significantly higher than visit 1 (Fig. 4) and the frequency of HIV p24− cells at visit 3 was significantly higher than visit 1 in black, but not Hispanic, women (Fig. 5). Taken together, our results indicate...
FIGURE 6. Immunological phenotypes of CD4⁺ T cells preferentially infected by HIV.
PBMCs exposed to HIV-1BaL (MOI 0.01) were cultured for 10 d. In HIV-infected PBMCs, cell surface marker expression of integrin α4β7, CCR5, and CD38 (A–C) and T cell subsets (D) were measured on HIV p24⁺ CD4⁺ T cells and HIV p24⁻ CD4⁺ T cells. Uninfected cells were included as a comparison. Each dot represents one donor. Bars represent median and IQR. The Mann–Whitney U test was used to compare cell surface marker expression between uninfected, HIV p24⁺, or HIV p24⁻ CD4⁺ T cells. A p value ≤ 0.05 was considered significant, p > 0.05 was not significant (ns).
Depo-Provera altered the immune markers associated with preferential HIV infection and increased HIV infection ex vivo. These changes varied depending on race and ethnicity, and the sample collection time after treatment.

Unlike studies in Depo-Provera–treated rhesus macaques that show a correlation between CD38+ T cell levels and HIV-RNA levels, suggesting CD38 may be an indicator of viral replication (35–37), we did not find a positive correlation between the frequency of integrin α4β7−, CCR5−, or CD38-expressing cells and HIV susceptibility (e.g., HIV p24+ cell numbers or HIV production). We did find a significant, but weak, negative correlation between HIV production at day 7 and cell surface expression of integrin α4β7 (r = −0.22, p = 0.038) and CD38 (r = −0.21, p = 0.04) on freshly isolated PBMCs (day 0) (Supplemental Fig. 3), but no significant correlation between markers on day 0 and the frequency of p24+ cells measured at day 10 postinfection. There was a weak negative correlation between HIV production and CD38 on freshly isolated PBMCs from black women (r = −0.3168, p = 0.0196). There was no significant correlation between HIV infection (HIV p24 release or p24+ cells) and the frequency of immune markers on PBMCs at day 10 postinfection except a very weak negative association between the frequency of integrin α4β7 and HIV production in Hispanic women (r = −0.38, p = 0.02) (Supplemental Fig. 4). The weak negative correlation between HIV infection and integrin α4β7 or CD38 may be attributed to increased cell death in the HIV-preferable cell populations that are highly infected by HIV. Detailed analyses of the dynamics between HIV infection and cell death with a sensitive method in these highly HIV susceptible cells would offer insights into HIV pathogenesis.

We found a significant increase in HIV preference markers at visit 2, but the increase in HIV infection was found at visit 3 compared with visit 1. The increase in HIV infection and preference markers at different visits may be due to a complex interaction of Depo-Provera with various immune parameters that modulate HIV infection. For example, other regulatory cells or soluble factors in PBMCs could be involved in Depo-Provera–mediated enhancement of HIV susceptibility at the visit 3. Depo-Provera use has been shown to alter pDC function with a decreased production of IFN-α and TNF-α in response to TLR-9 stimulation (29). HIV host restriction factors such as IFN-inducible cellular proteins can block multiple stages of the HIV lifecycle (38, 39). Changes in cytokine levels induced by Depo-Provera may also modify levels of intrinsic host restriction factors in CD4+ T cells, resulting in a decreased ability of CD4+ T cells to control HIV infection. Some immunomodulatory impacts on HIV infection may not be observed until the decreased concentration of Depo-Provera at visit 3.

In the HIV infection ex vivo assay, we found a significant increase in HIV infection from visit 1 to visit 3 when analyzing HIV p24 production at day 7 postinfection (Fig. 4A) but not the frequency of HIV p24+ cells at day 10 postinfection (Fig. 5A). Similarly, the difference in the frequency of HIV p24+ cells at day 10 postinfection in black women in response to Depo-Provera (Fig. 5B) was not found in the HIV p24 production at day 7 postinfection (Fig. 4B). Multiple elements may contribute to the discrepancy between the results of two assays (HIV p24 ELISA versus HIV p24+ flow cytometry) in a multicellular system for HIV infection of PBMCs ex vivo. These factors include 1) the impact of...
Depo-Provera on other immune cells (e.g., monocytes or pDCs) or metabolites that could affect CD4+ T cells, the dynamics between HIV and PBMCs (e.g., compositions of immune cell types, cell activation or death state, and cytokine production), and 3) the biological variability in women and subjects’ metabolisms in response to Depo-Provera. Future studies using purified CD4+ Tcells for HIV infection assay and determining the functions of other immune cells or immune milieu in clinical samples may offer insights into development a better HIV ex vivo assay to predict clinical outcomes.

Our results revealed ethnicity-dependent changes to immune profiles and HIV susceptibility in response to Depo-Provera. For example, we observed an increase in integrin α4β7 expression from visit 1 to visit 2 in Hispanic women, with no significant change in expression from visit 2 to visit 3. In black women, there was an increasing trend of integrin α4β7 expression from visit 1 to visit 2 but the level decreased from visit 2 to visit 3. Significant decreases in TEM cells from visit 1 to visit 2 and visit 1 to visit 3 were observed only within the black patient group. Additionally, black women had a significant increase in the percentage of p24+ cells from visit 1 to visit 3, whereas the Hispanic patient group showed no significant difference in the number of infected cells between visits. Although there was a difference in the smoking status or body mass index (BMI) between these two groups [smoking, Hispanic: 1 out of 15, black: 8 out of 24; BMI (median), Hispanic: 25.27, black: 30.3], our conclusion remains the same after stratifying for smoking and BMI. Race and ethnicity are known to contribute to disease susceptibility and vaccine responses (40, 41). Blacks comprise 12.3% of the United States population but accounted for 44.3% of new HIV diagnoses whereas Hispanics, who comprise 17% of the United States population but accounted for 44.3% of new HIV diagnoses in 2015 (42). Although racial and ethnic disparities in HIV transmission are likely multifactorial (43–45), the role of race- and ethnicity-dependent immune responses in modulation of HIV transmission requires further investigation.

A recent longitudinal study by Achilles indicates no change in CCR5 or CD69 (activation marker) on peripheral or cervical CD4+ T cells in women 90 d after initiation of Depo-Provera (46). However, a cross-sectional study performed by Sciaranghella detected significantly higher CCR5 MFI on CD4+ T cells from Depo-Provera users compared with a control group without hormonal contraception (28). We found a significant increase in integrin α4β7+CD4+ T cells, and an increasing trend in CCR5+CD4+ T cells 1 mo after Depo-Provera injection compared with baseline or 3 mo after Depo-Provera injection. There was no significant change in these markers on CD4+ T cells between the baseline first visit and 3 mo (90 d) after Depo-Provera. These results suggest that study design, particularly the timeframe when samples are collected, may impact the outcome. Weinberg et al. (30) found a significant decrease in activated CD4+ T cell subsets (CD4+CD25– T cells) 3 mo after Depo-Provera injection in HIV-infected women on cART, whereas we observed a trend of increased CD38+CD4+ T cells at visit 2 but not visit 3. It is not clear whether the different results are due to the status of HIV, cART, age, or race and ethnicity in these two studies.

In addition to PBMCs, immune cells within the female genital tract are impacted in response to Depo-Provera. Depo-Provera users have higher levels of activated endometrial T cells along with a higher percentage of endometrial CD4+ naive and CM subsets compared with women not using hormonal contraceptives, although there is no difference in their TEM subsets (47). We observed a similar increasing trend of naive and CM CD4+ T cells subsets in response to Depo-Provera, especially in black patients, indicating changes in CD4+ T cell subsets by Depo-Provera may not be compartmentalized. However, our longitudinal study revealed a decreasing trend of TEM cells in response to Depo-Provera, with a significant reduction observed within the black patient group. It remains to be determined whether Depo-Provera-mediated alteration of the TEM cell population is location dependent (blood versus mucosal tissues).

Although Depo-Provera appeared to have a sustained effect on CD4+ T cell subsets, we found a transient increase in HIV preference markers at visit 2 (1 mo) followed by a decrease to baseline levels at visit 3 (apart from expression of integrin α4β7 in Hispanic women). This finding differs from a study comparing immune cells in vaginal tissues before and 12 wk (the same timing as visit 3) after receiving Depo-Provera, which showed Depo-Provera treatment resulted in a significant increase in the median numbers of CD45s, CD3s, CD8s, CD68s, CCR5s, and HLA-DR–positive cells regardless of whether samples were collected at the follicular or luteal phases (48). It is possible that cells at the mucosal sites are regulated differentially from peripheral cells, and are less dependent on serum concentrations of Depo-Provera.

In summary, our longitudinal study demonstrated that Depo-Provera changed markers for HIV preference and CD4+ T cell subsets in PBMCs and increased HIV infection ex vivo despite a small sample size. Our results warrant future investigation on the mechanism of Depo-Provera–mediated increased HIV preference markers such as integrin α4β7. We showed differential immune profiles and HIV infection could vary depending on sampling time and the race and ethnicity of study subjects, which may explain the inconsistent findings of the association of Depo-Provera and increased risk of HIV acquisition. The impact of Depo-Provera on immune homing markers and T cell subsets suggests that Depo-Provera may impact the establishment of HIV reservoirs and immune regulation during HIV pathogenesis (49, 50). Thus, a better understanding of how Depo-Provera modulates immune responses in women with diverse backgrounds and health states is critical for developing an effective strategy for HIV prevention and treatment.

**DISCLOSURES**

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