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EFFECT OF DEPOT MEDOXYPROGESTERONE ACETATE ON IMMUNE FUNCTIONS AND INFLAMMATORY MARKERS OF HIV-INFECTED WOMEN

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Abstract

Objectives—Depot medroxyprogesterone acetate (DMPA) was associated with increased HIV transmission and accelerated disease progression in untreated women. The potential underlying mechanisms include immune-modulation. We evaluated the effect of a single DMPA injection on cell-mediated immunity (CMI), T cell activation (Tact), regulation (Treg) and inflammation in HIV-infected women on cART.

Methods—Women with HIV plasma RNA 400 c/mL on stable cART received DMPA and had immunologic and MPA measurements at baseline, 4 weeks [peak MPA concentration (Cmax)] and 12 weeks [highest MPA area under the concentration curve (AUC)].

Results—At baseline, among 24 women with median 32 years of age and 622 CD4+ cells/μL, 68% had HIV, VZV, PHA and CD3/CD28 CMI measured by lymphocyte proliferation and/or IFNγ/IL2 dual-color fluorospot. CMI did not significantly change after DMPA administration except for a 1.4-fold increase in IL2/IFNγ VZV fluorospot at week 12. Tact decreased after DMPA administration, reaching statistical significance at week 12 for CD4+CD25+%. Treg behaved heterogeneously with an increase in CD8+FOXP3+% at week 4 and a decrease in CD4+IL35+% at week 12. There was a decrease in TGFβ at week 12 and no other changes in plasma biomarkers. Correlation analyses showed that high MPA Cmax and/or AUC were

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significantly associated with increases of IFNγ HIV ELISPOT, CD4+IL35+% and CD4+TGFβ+% Treg and decreases of plasma IL10 from baseline to weeks 4 and/or 12.

Conclusions—A single dose of DMPA did not have immune-suppressive or pro-inflammatory effects in HIV-infected women on cART. Additional studies need to assess the effect of multiple doses.

Introduction

Ensuring access to preferred contraceptive methods for women and couples is essential to securing the well-being and autonomy of women [1, 2]. This aspect is particularly relevant to HIV infection, where family planning reduces the risk of unintended pregnancies among women living with HIV. Although the strategies to prevent mother-to-child HIV transmission have been extremely successful, there is still a limited risk of intra uterine and breast milk HIV transmission and also a risk of children being orphaned due to premature maternal death that could be prevented by adequate contraception. Among the different methods of contraception, condoms are highly recommended, because they have the advantage of providing a physical barrier against sexually transmitted infections, including HIV. However, the effectiveness of condoms as contraceptives is <80%, whereas hormonal contraceptives (HC) have an effectiveness of 94% and 91% for injectable and oral preparations, respectively [1]. In addition, compared to condoms, the use of HC is more readily controlled by women conferring them a degree of choice and autonomy.

In the context of HIV infection, the use of HC has been hotly debated. There is evidence that the use of HC may increase the risk of HIV transmission, acquisition and may accelerate progression of HIV infection in women who are not on antiretroviral (ARV) therapy [3–12]. In addition, compared with HIV-infected women using non-hormonal birth control methods, HIV-infected women on HC had higher HIV loads in genital secretions [13]. Pregnancy, a state of heightened female hormone secretion, has also been associated with modest increases both in the risk of HIV acquisition and in HIV loads in the genital tract [14, 15]. The results of these studies might have been influenced by socioeconomic and behavioral characteristics of the study population that are difficult to separate from those directly generated by the use of HC on the risk of HIV acquisition, transmission or progression. However, studies of HC in animal models of simian immunodeficiency virus (SIV) or simian-HIV (SHIV) acquisition also found an increased risk of infection in animals treated with supraphysiologic doses of female hormones [16, 17]. It should be noted that the effect of hormonal contraceptives on transmission of HIV or other primate lentiviruses and on disease progression remains controversial, with multiple studies failing to demonstrate any associations [4, 5, 18–23].

Human and animal studies sought to elucidate the mechanism(s) that mediate the potential increased risk of HIV infection and disease progression associated with the use of HC. These ultimately found two important, non-mutually exclusive potential mechanisms: 1) changes in the female reproductive tract [24–28] and 2) attenuation of cell-mediated immunity (CMI) [16, 17, 29–33].

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Among the HC, depot medoxyprogesterone acetate (DMPA) is one of the most widely used worldwide including areas of high prevalence of HIV infection, such as sub-Saharan Africa and Southeast Asia [34]. In the US, women enrolled in the Women's Interagency HIV Study reported roughly equal use of DMPA and oral HC [35]. DMPA is administered intra-muscularly every 3 months, ensuring better compliance compared with oral HC, which typically require daily administration. Furthermore, DMPA maintains effectiveness when co-administered with efavirenz, which typically decreases the effectiveness of progestin implants [36]. However, DMPA is also commonly used to promote female genital tract infection with SHIV and SIV in non-human primates taking advantage of its local effects on the female reproductive tract [16]. It is also purported to depress CMI through its dual action on progestin and glucocorticoid receptors, which are widely expressed by lymphocytes and other mononuclear cells [37–41].

In this study, we investigated the CMI in participants of the AIDS Clinical Trials Group (ACTG) study A5283 [42]. The goal of the immunologic component of the study was to compare functional CMI against HIV and varicella-zoster virus (VZV), T cell activation and regulation, and inflammatory biomarkers before and after DMPA administration and to examine potential associations of immunologic and inflammatory characteristics with medroxyprogesterone acetate (MPA) plasma concentrations.

Subjects and methods

Study Design

The study was a 12-week, multicenter, open-label, non-randomized trial in which a single dose of 150 mg DMPA was administered intramuscularly to non-pregnant, pre-menopausal HIV-1-infected women   15-year old, who did not use DMPA for at least 6 months prior to enrollment and were on a stable combination antiretroviral regimen (cART) containing lopinavir with ritonavir boost (LPV/r) administered twice daily for at least 12 weeks prior to enrollment. All subjects had plasma HIV-1 RNA   400 copies/mL within 30 days of study entry and were required to continue on cART for the 12 weeks of the study. Blood samples for MPA and progesterone concentrations, plasma HIV RNA levels, CD4+ cell counts and immunologic and inflammatory measurements were collected at study entry (week 0, pre-DMPA administration), and 4 (putative peak of MPA) and 12 (putative trough of MPA) weeks after DMPA administration.

Processing of samples for Immunologic Assays

Peripheral blood mononuclear cells (PBMC) were cryopreserved at the clinical site laboratories following a standardized protocol (http://www.hanc.info/labs/Pages/SOPs.aspx). All laboratories were in good standing with the Immunology Cryopreservation Quality Assurance program [43]. Cryopreserved PBMC were shipped within 7 days of collection to a central repository where cells were stored in liquid N_2 until shipment in liquid N_2 dewars to the testing laboratory at the University of Colorado Anschutz Medical Center. This procedure ensured optimal viability and functionality of the PBMC [44]. Plasma was separated and cryopreserved in 1 mL aliquots by the processing laboratories and

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batch-shipped on dry ice. All specimens from each subject were tested in the same run to avoid confounders potentially introduced by inter-assay variability.

Fluorospot

Dual-color fluorospot kits for IFNγ and IL2 (Mabtech) were used as per manufacturer's instructions. PBMC were thawed and rested over night. Cells with viability 70% before and after resting were added at 250,000 PBMC/well in 100 μL of RPMI 1640 with glutamine (Gibco) containing 10% human AB serum (Nabi) and 1% antibiotics (Gibco) and stimulated in duplicate wells with HIV inactivated virions and control (gift of Dr. Jeff Lifson [45]; 6 μg/mL), VZV inactivated cell lysate and mock-infected control prepared as previously described [46] at a pre-optimized concentration, phytohemagglutinin A (PHA; Sigma; 0.01 μg/mL) or anti-CD3 and anti-CD28 mAb (CD3/CD28; Mabtech; 0.1 μg/mL). After 36 h at 37°C in a 5% CO_2 humidified atmosphere, plates were washed; bound IFNγ was detected with 7-B6-1-FS FITC and bound IL-2 with 11-Biotin. Spots were revealed using a mixture of anti-FITC-Green fluorochrome (IFNγ) and SA-Red fluorochrome (IL-2) and analyzed with an Immunospot II plate reader (CTL). Results were reported as mean spot-forming cells (SFC)/10^5 PBMC in antigen- or mitogen-stimulated wells after subtraction of the mean SFC in control wells.

Lymphocyte proliferation assay (LPA)

The LPA was performed on freshly thawed PBMC with a viability 70% as previously described [47]. Stimulants consisted of HIV antigen and control (1.5 μg/mL), VZV antigen and control (0.1 μg/mL), CD3/CD28 (0.1 μg/mL) and PHA (2.5 μg/mL). Results are presented as stimulation indices (SI) calculated by dividing the median counts per minute (cpm) in the antigen-stimulated wells, by the median cpm in the control wells.

Flow cytometry assays

T-cell subsets were enumerated in freshly thawed cryopreserved PBMC. After washing and counting viable cells, PBMCs were surface-stained with the following conjugated mAbs: anti-CD3-AF488 (Biolegend; clone HIT3a), anti CD4-APC/Cy7 (Biolegend; RPA-T4), anti-CD25-PE/Cy7 (Biolegend; BC96), anti-HLA-DR-PerCP/Cy5.5 (Biolegend; L243), anti-CD39-APC (Biolegend; A1), and anti-CD38-PECy7 (Biolegend; HIT2). Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with anti-IL10-APC (R & D Systems; 127107), anti-FOXP3-PE (Biolegend; 206D), anti-TGFβ-PerCP/Cy5.5 (Biolegend; TW4-2F8) and anti-IL35-PE (eBioscience; ebic6) and analyzed with Guava easyCyte 8HT and FlowJo (Treestar).

Subsets were expressed as percentages of the parent CD4+ and CD8+ T-cell populations. The gating strategy is presented in Fig S1.

Soluble cytokines

IL6, IL8, IL10, IFNγ and TNFα plasma levels were measured by multiplex bead array and TGFβ by ELISA as previously described [48]. The bead array assays used the MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel kit (Millipore; HSCYTMAG-60SK) on the Bio-Rad Bio-Plex 200 instrument following the manufacturers'

instructions. The lower level of detection (LLOD) was 0.08–1.01 pg/mL and the dynamic range 13–2,000 pg/mL. Data were analyzed using Bio-Plex manager 5.0 software (Bio Rad) and concentrations were interpolated on the manufacturer's standard curve using PRISM software (Graphpad). The LLOD for the TGFβ ELISA was 31 pg/mL and the dynamic range 31–4,000 pg/mL. The optical density (OD) was measured with a Multiscan FC ELISA reader (Thermo Fisher) using a 450 nm filter. Test TGFβ concentrations were calculated by interpolating the test ODs on the standard curve built with quantitative controls provided by the manufacturer.

Statistical analysis

All analyses were conducted on subjects with assay results available for immunologic and inflammatory endpoints using non-parametric statistical approaches, unless otherwise noted. The effects of hormonal contraceptive MPA on those immunologic markers were evaluated based on intra-subject changes using nonparametric Wilcoxon signed-rank test. Spearman's correlation test was used to assess associations between immunologic biomarkers and associations of immunologic markers against MPA pharmacokinetic parameters. All analyses were conducted using SAS (SAS Institute Inc.).

Results

Characteristics of the study population

The study used blood samples from 24 HIV-infected women with a median of 32 years of age (Table 1). As per inclusion criteria, all participants had HIV plasma RNA   400 copies/mL on LPV/r-containing cART. CD4+ cell numbers varied from 326 to 1367 cells/μL (median=622 cells/μL) at study entry.

Functional cell-mediated immunity of HIV-infected women before and after a single dose of DMPA

At entry, women had robust HIV-, VZV-, PHA- and CD3/CD28-stimulated LPA responses (Fig 1), including median (interquartile ranges; IQR) LPA SI for HIV of 51 (5, 74), for VZV 22 (3, 67), for CD3/CD28 65 (11, 135) and for PHA 105 (33, 166). Using a threshold   3 for HIV and VZV and   5 for CD3/CD28 and PHA to define positive results, 78% had positive qualitative results for HIV, 77% for VZV, 86% for CD3/CD28 and 90% for PHA. There were no significant quantitative or qualitative changes in the LPA responses after DMPA administration (Fig 1).

IFNγ FLUOROSPOT results at entry showed median (IQR) SFC/10^5 PBMC of 83 (4, 152) for HIV, 3 (1, 12) for VZV, 133 (47, 233) for CD3/CD28 and 133 (34, 221) for PHA (Fig 2A). At 4 and 12 weeks after DMPA administration, HIV, CD3/CD28 and PHA IFNγ SFC did not appreciably change compared to baseline (Fig 2A). VZV IFNγ SFC did not change from baseline to week 4, but increased at week 12 by a median of 2 SFC/10^5 PBMC (fold-rise = 1.4, $p = 0.007$). IL2 SFC were highly correlated with those of IFNγ and showed similar changes or lack thereof over time (Fig 2B).

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CD4+ and CD8+ T cell subsets of HIV-infected women before and after a single dose of DMPA

Activated T cells (Tact) were identified by the expression of CD25 and by dual expression of CD38 and HLADR (Table 2). There were small changes in the distribution of activated T cell subsets in the course of the study including a median decrease of 0.5% in CD4+CD25+% at week 12 that reached statistical significance (from a median of 3.42% to 2.84%, p=0.03); and lesser decreases at week 12 of 0.05% in CD8+CD25+% (from 0.39% to 0.34%; p=0.08) and 0.7% in CD8+CD38+HLADR+% (from 4.47% to 3.79%, p = 0.06).

Regulatory T cell subsets were characterized by expression of FOXP3, dual expression of CD25 and FOXP3, CD39, IL10, IL35 and TGFβ (Table 2). At week 4 after DMPA administration, there was a small, but statistically significant increase of 0.06% in CD8+FOXP3+% (from 0.85% to 0.91%, p=0.02). At week 12, there was a statistically significant decrease of 0.5% in CD4+IL35+% (from 1.86% to 1.35%, p=0.02) and a trend increase of 1.5% in CD4+CD39+% (from 10.1% to 11.6%, p=0.08).

Soluble biomarkers of HIV-infected women before and after a single dose of DMPA

Pregnancy, which is a state of heightened female hormone secretion, is also characterized by increased production of inflammatory and regulatory cytokines [49]. Pro-inflammatory (IL6, IL8, IFNγ and TNFα) and regulatory cytokines (IL10 and TGFβ) were measured before and after DMPA administration. There were no significant changes over time in plasma concentrations of IL6, IL8, IL10, IFNγ or TNFα. TGFβ had a small, but significant decrease at week 12 (p=0.04).

Relationship between MPA plasma concentrations and immunologic parameters in HIV-infected women

Correlation analyses were performed to determine the relationship of MPA C_{max} and AUC with changes in functional immune responses (ratios of post-DMPA over baseline results), T cell subsets (differences in percentages from baseline to post-DMPA) and/or plasma biomarkers (ratios of post-DMPA over baseline results). High MPA C_{max} were associated with increased HIV IFNγ FLUOROSPOT fold-rises at week 12 over baseline (r=0.64, p=0.003; Fig 3), CD3/CD28 IFNγ at week 4 (r= 0.51, p= 0.04; Fig 3), CD4+IL35+% at week 4 and 12 (r 0.51, p 0.02; Fig 3) and CD4+TGFβ+% at week 4 (r=0.44, p=0.04; Fig 3). In addition, high MPA AUC was associated with increases in CD4+IL35+% at week 4 and 12 compared to baseline (r=0.47, p=0.03 for both; Fig 3) and with a decrease in the plasma concentration of IL10 (r=−0.46, p=0.03; Fig 3).

Discussion

This exploratory analysis of the effect of DMPA on CMI of HIV-infected women on effective cART failed to show any attenuation of their CMI after a single dose of DMPA. This result was unexpected based on the accumulated evidence that suggested a downregulatory and anti-inflammatory effect of progestins in general and of MPA in particular on CMI [16, 17, 29, 33, 37–40, 50–54]. In contrast to these studies, we observed an increase in VZV-specific IL2 and IFNγ FLUOROSPOT responses after DMPA

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administration compared to baseline. In addition, we found positive associations of the MPA Cmax with the fold-increase of HIV-specific IFNγ responses from baseline to week 12 and of CD3/CD28-stimulated IFNγ responses from baseline to week 4. A mechanism to explain the association of DMPA administration with the CMI increases that we observed is unclear. Based on previous in vitro data, it is unlikely that it involved a direct effect of MPA on the cells of the immune system. However, an indirect effect mediated by the decrease of estrogen secretion in DMPA recipients [55] is a potential explanation due to the strong immune regulatory effect of estrogen [56–66].

It is important to note that our study evaluated women on effective cART, a characteristic shared by other studies that failed to detect any detrimental effects of DMPA on HIV disease progression or transmission studies [18, 21, 67]. In contrast, studies that documented detrimental effects of DMPA were generally conducted in the absence of cART [7, 8]. A stimulatory direct effect of MPA and of other steroidal hormones on HIV replication has been described [68]. The use of cART might attenuate the direct effect of MPA on HIV replication thus contributing to the difference in findings between DMPA studies conducted in the presence or absence of cART. If the deleterious effect of MPA on the CMI of HIV-infected women is indeed mediated by the enhancement of HIV replication, the current trend towards increased cART utilization may improve the safety of DMPA administration to HIV-infected women.

T-cell activation markers decreased after DMPA administration, an effect that was predicted by in vitro studies showing that MPA decreased dendritic cell and monocyte-activation and production of pro-inflammatory cytokines [30, 31, 33, 39, 40]. However, we were not able to demonstrate a decrease in pro-inflammatory cytokine plasma levels. A recent study showed increased T-cell activation in HIV-uninfected women receiving DMPA compared with women using oral or no hormonal contraceptives [69]. The divergence may be related to differences in study populations, HIV-infected vs. uninfected, and designs, longitudinal vs. cross-sectional. The longitudinal study design allowed us to use the subjects as their own controls, thus avoiding potential biases introduced by differences in exposures to other factors that may promote T-cell activation.

Based on previous studies [53, 54], we expected an increase in circulating Treg subsets after DMPA administration. In fact, we observed a correlation between high MPA Cmax and AUC with an increase in CD4+IL35+% and in CD4+TGFβ+% after DMPA administration compared to baseline, which was consistent with a stimulatory effect of MPA on Tregs. However, the changes in the proportion of Tregs were heterogeneous among study participants and at the group level, there was no consistent increase in the proportion of Tregs over time on DMPA. This suggests that the stimulatory effect of MPA on Treg subsets may be offset by the DMPA-mediated decrease in estrogen, which is also a Treg inducer, perhaps even more potent than MPA [64, 65].

Our study had limitations and virtues. Because this was an exploratory analysis we did not adjust for multiple comparisons. This increased our ability to detect changes and generate hypotheses, but might have also allowed the introduction of spurious associations. Although the number of participants in this study was limited, their ages, which varied from 15 to 47

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years, overlap with the age range of 15 to 44 years considered by the CDC as representative for the use of contraceptive methods in the U.S. and with previously published demographics of DMPA in HIV-infected users [70, 71]. The limited number of participants in this analysis was counterbalanced by the longitudinal design using intra-subject comparison approaches, which minimized the introduction of DMPA-unrelated variables. Furthermore, longitudinal samples of each participant were assayed jointly in order to minimize the effect of inter-assay variability.

Although our findings need to be confirmed, these preliminary results are encouraging, because they support the notion that the effectiveness of DMPA as a contraceptive agent in the context of cART overpowers its potential attenuation of immune defenses in HIV-infected women. Our results also show that the effect of in vivo administration of hormones have consequences that may not be anticipated from their effect in vitro, probably due to feed back mechanisms that are intact in vivo and change the overall hormonal homeostasis and cannot be readily reproduced in vitro. More studies are needed to evaluate the cumulative effect of multiple doses of DMPA on the immune system of HIV-infected women and its effect on HIV transmission.

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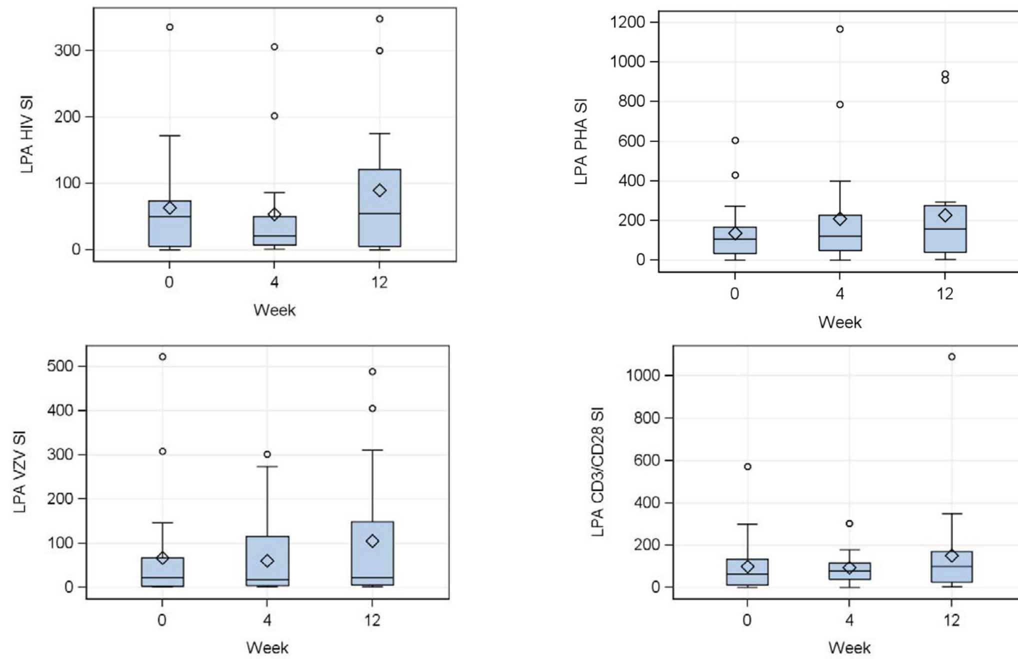

Figure 1. Effect of a single dose of DMPA on lymphocyte proliferative responses in HIV-infected women

Data were derived from 24 HIV-infected women. Proliferation was measured by ^{3}H-Thymidine incorporation after 6 days of in vitro stimulation of PBMC collected at the indicated time points. Stimulation indices (SI) calculated by the median incorporation in stimulated PBMC divided by the median incorporation in unstimulated or mock-stimulated controls, for HIV antigen (upper left), VZV antigen (lower left), PHA mitogen (upper right) and CD3/CD28 ligands (lower right) are summarized in box plots showing the medians as horizontal lines inside the boxes; means as diamonds inside the boxes; upper and lower quartiles as the box boundaries; minimum and maximum values, excluding potential outliers, as whiskers and outliers as open circles. There were no significant changes from week 0 (DMPA administration) to week 4 (peak MPA concentration) or week 12 (maximum exposure to MPA).

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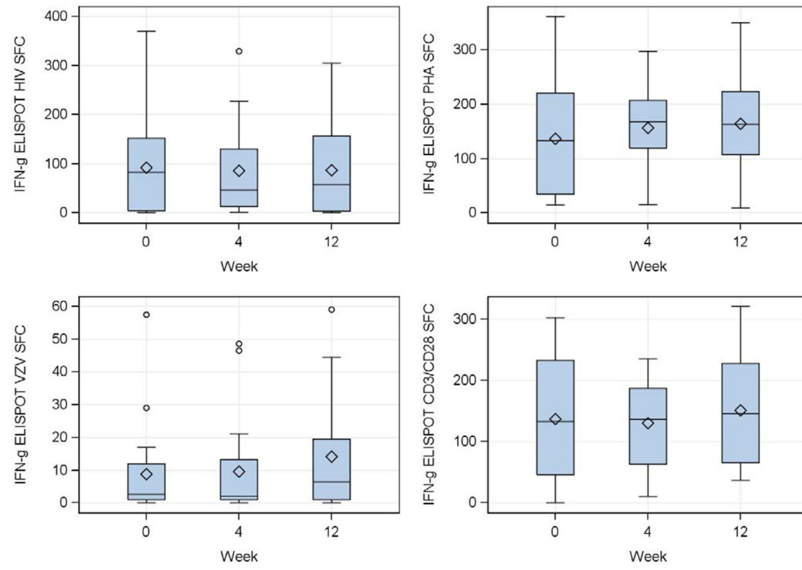

Figure 2A

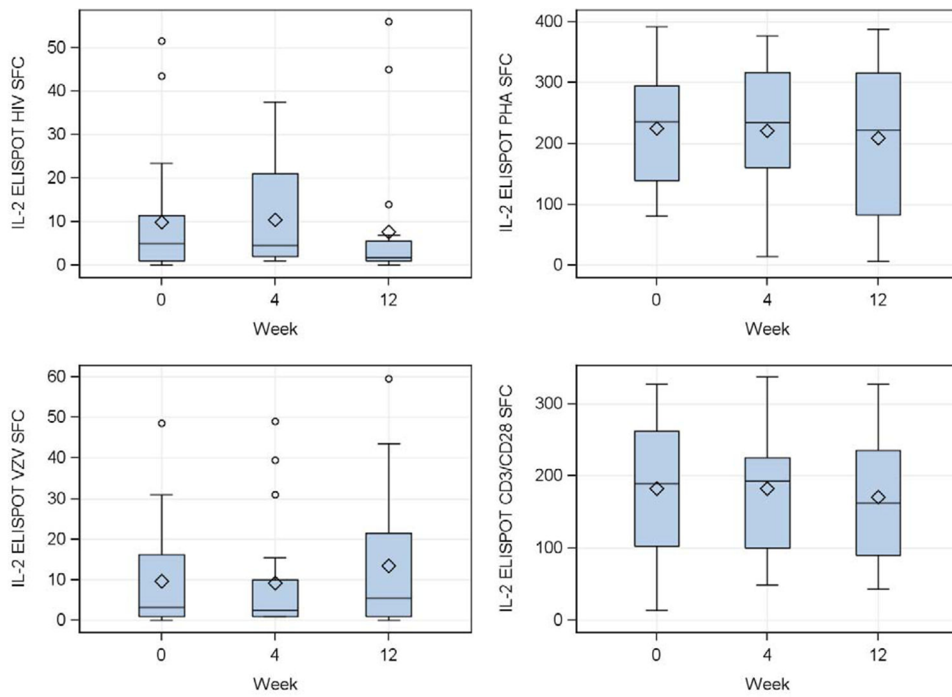

Figure 2B

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Figure 2. Effect of a single dose of DMPA on ELISPOT responses in HIV-infected women

Data were derived from 24 HIV-infected women. Interferon γ (IFNg; **Panel A**) and IL2 secretion (**Panel B**) were measured by dual-color fluorospot. Adjusted spot forming cells (SFC)/10^5 PBMC calculated by subtraction of the mean SFC in control unstimulated or mock-stimulated wells from the mean SFC in stimulated PBMC, for HIV antigen (upper left), VZV antigen (lower left), PHA mitogen (upper right) and CD3/CD28 ligands (lower right) are summarized in box plots showing the medians as horizontal lines inside the boxes; means as losanges inside the boxes; upper and lower quartiles as the box boundaries; minimum and maximum values, excluding potential outliers, as whiskers and outliers as open circles. There were significant increases from week 0 (DMPA administration) to week 12 (maximum exposure to MPA) in VZV IFNγ and IL2 SFC (p=0.007 for both). SFC in other conditions remained unchanged.

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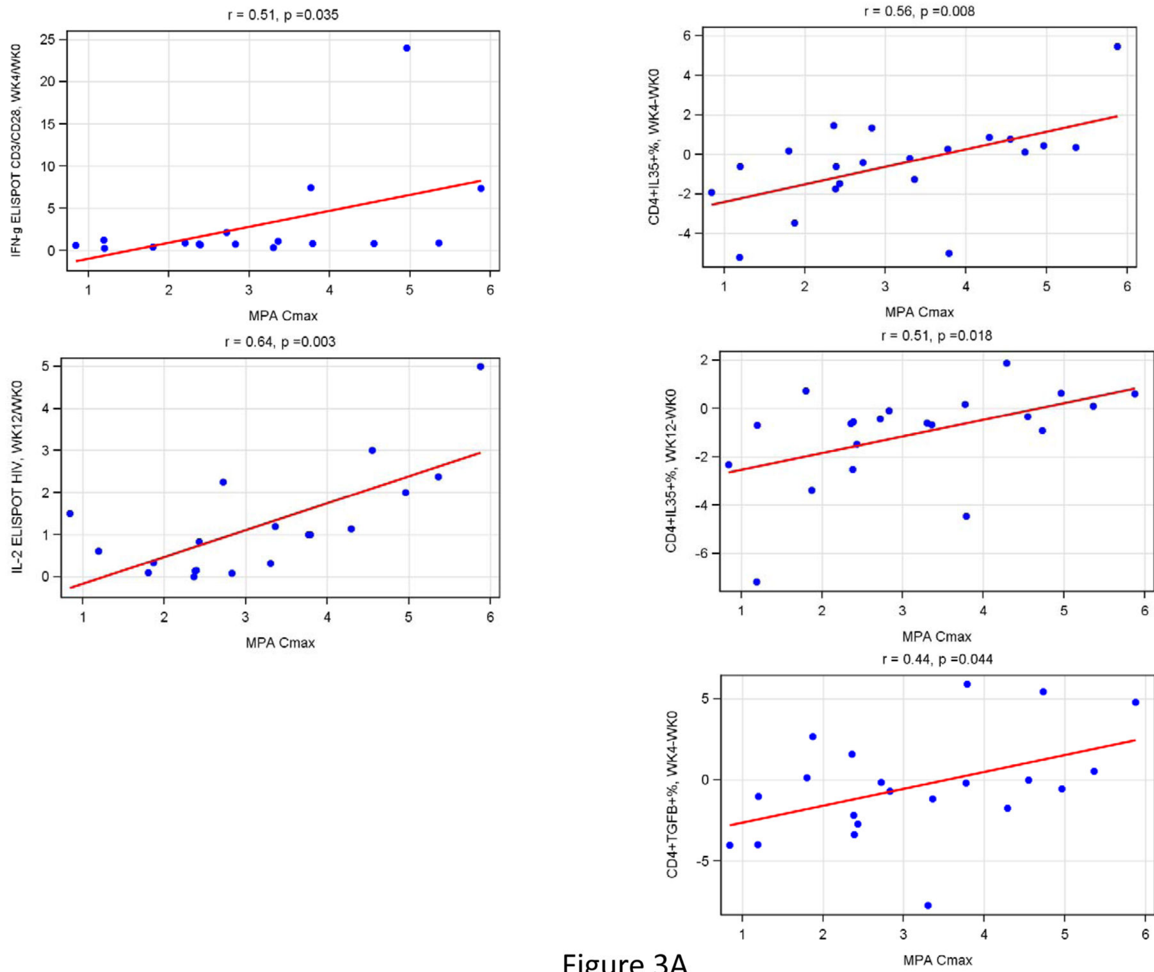

Figure 3A

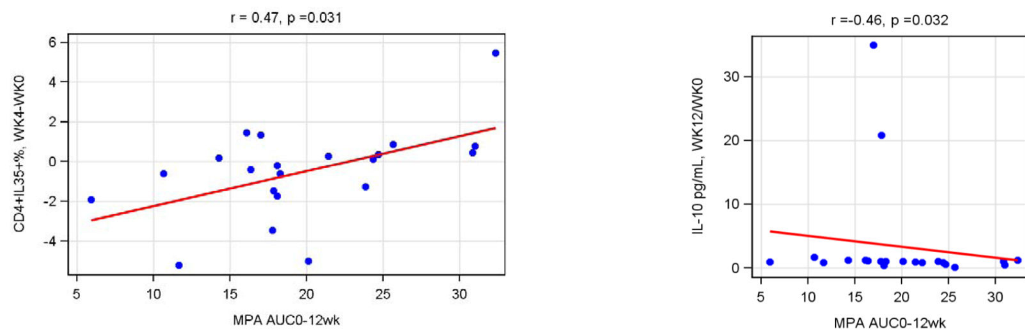

Figure 3B

Figure 3. Effect of the magnitude of MPA Cmax (Panel A) and T-cell subsets (Panel B) on CMI, T-cell subsets and soluble cytokines in HIV-Infected women

Data were derived from 24 HIV-infected women who received a single dose of DMPA. MPA C_{max} was measured at week 4 and AUC at week 12 after DMPA administration. Correlation analyses were performed between changes from baseline to week 4 or 12 of all

the measures of CMI, T-cell subsets and soluble cytokines evaluated in this study. The graphs show only the significant correlations. Estimated linear regression lines, correlation coefficients (r) and p values calculated using Spearman's correlation test are shown on each graph.

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Table 1

Baseline Characteristics of the Study Group

Characteristics	**N = 24**
Years of age: median [range]	32 [15–47]
Race/ethnicity, n (%)	
White	3 (13%)
Black	14 (58%)
Hispanic	6 (25%)
Native American	1 (4%)
Median CD4+ cells/µL [range]	622 [326, 1367]
HIV-1 plasma RNA 400 copies/mL, n (%)	24 (100%)

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Table 2

Kinetics of T-cell subsets after a single dose of DMPA in HIV-infected women on effective cART.

Anchor	T-cell subset	Week	N	Median (%)	IQR (Q1, Q3)	p-value[1]
CD4	**CD4+CD25+**	0	21	3.42	(2.02, 5.05)	
		4	21	2.85	(1.73, 6.25)	0.533
		12	21	2.84	(1.63, 4.33)	**0.029**
	CD4+FOXP3+	0	21	1.26	(0.80, 2.73)	
		4	21	1.30	(0.90, 1.46)	0.828
		12	21	1.29	(1.01, 1.80)	0.511
	CD4+CD25+FOXP3+	0	21	0.41	(0.17, 0.80)	
		4	21	0.24	(0.11, 0.62)	0.176
		12	21	0.33	(0.13, 0.45)	0.100
	CD4+CD39+	0	21	10.1	(5.95, 15.9)	
		4	21	10.1	(6.31, 13.2)	0.763
		12	21	11.6	(7.65, 16.5)	**0.078**
	CD4+CD38+HLADR+	0	21	7.52	(4.19, 9.55)	
		4	21	5.98	(4.05, 10.1)	0.206
		12	21	5.65	(3.35, 9.39)	0.269
	CD4+TGFB+	0	21	4.32	(2.66, 6.02)	
		4	21	3.62	(1.93, 7.96)	0.334
		12	21	3.08	(2.30, 5.62)	0.308
	CD4+IL10+	0	21	4.66	(3.52, 6.16)	
		4	21	6.01	(3.41, 7.68)	0.511
		12	21	5.64	(3.56, 8.43)	0.427
	CD4+IL35+	0	21	1.86	(1.22, 3.36)	
		4	21	1.59	(0.93, 3.19)	0.352
		12	21	1.35	(0.78, 2.08)	**0.024**
CD8	**CD8+CD25+**	0	21	0.39	(0.23, 0.48)	
		4	21	0.38	(0.22, 0.57)	0.427
		12	21	0.34	(0.14, 0.41)	**0.078**
	CD8+FOXP3+	0	21	0.85	(0.54, 1.04)	

Anchor	T-cell subset	Week	N	Median (%)	IQR (Q1, Q3)	p-value[1]
		4	21	0.91	(0.68, 1.49)	**0.023**
		12	21	0.74	(0.67, 1.43)	0.176
	CD8+CD25+FOXP3+	0	21	0.07	(0.03, 0.19)	
		4	21	0.06	(0.03, 0.27)	0.388
		12	21	0.08	(0.01, 0.26)	0.556
	CD8+CD39+	0	21	3.38	(2.53, 4.52)	
		4	21	4.20	(2.37, 4.89)	0.848
		12	21	3.12	(2.31, 5.20)	0.675
	CD8+CD38+HLADR+	0	21	4.47	(2.35, 5.72)	
		4	21	4.28	(2.44, 7.26)	0.300
		12	21	3.79	(2.47, 5.55)	**0.056**
	CD8+TGFB+	0	21	1.26	(0.74, 1.90)	
		4	21	1.22	(0.76, 2.02)	0.159
		12	21	1.18	(0.60, 1.66)	0.404
	CD8+IL10+	0	21	1.46	(1.10, 1.87)	
		4	21	1.53	(1.13, 2.60)	0.290
		12	21	1.45	(1.10, 2.21)	0.701
	CD8+IL35+	0	21	1.22	(0.87, 1.61)	
		4	21	1.52	(0.53, 1.99)	0.490
		12	21	0.89	(0.65, 1.83)	0.500

[1] Wilcoxon signed-rank test of changes from baseline.

Bold font highlights significant differences and strong trends.

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